ULTRASTRUCTURAL STUDIES OF CONIDIOGENESIS IN *PSEUDOROBILLARDA PHRAGMITIS*

A THESIS

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ABSTRACT

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Ultrastructural Studies of Conidiogenesis in Pseudorobillarda phragmitis

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An ultrastructural study of conidia and conidiogenous cells of Pseudorobillarda phragmitis (Cunnell) Morelet in various stages of development is presented using light, phase, scanning and transmission electron microscopy.

Conidia formation has been observed to be enteroblastic. The conidiogenous cell arises as a small primordium from the tip of the basal cell. No distinction of a conidiogenous cell wall is observed during early conidiogenesis, and there is no discontinuity of plasmalemma and cytoplasm until delimited by a hyphal constriction. During conidiogenesis the conidiogenous cell increases in length with little increase in width. A conidium cross septum is not formed until conidial maturation and release.

Besides the usual cellular organelles, vesicles and concentric membrane organelles were observed. The occurrence and position of vesicles during early conidiogenesis support the assumption that these structures are associated with wall synthesis. Myelin figures were

observed after paraformaldehyde fixation and potassium permanganate postfixation. These structures may play different physiological roles according to their developmental stages and their locations. In this study they are not viewed as artifacts.

The setulae, which are formed after conidium release, are formed at the apical end of the conidium from strips of the conidiophore wall and not from strips of the outer walls of the spore as noted by others.

ACKNOWLEDGMENTS

The author expresses her appreciation and thanks to Dr. Lafayette Frederick for his excellent advice and assistance in the preparation of this thesis. I would also like to thank him for his unending patience and guidance during the progress of this research. Additional appreciation is extended to Mrs. Antoinette Frederick for the typing of this thesis. Thanks are also expressed to my sons, Walter Leonard Gray, Jr. and Daniel Allen Gray. Special thanks are extended to my husband, Walter Leonard Gray, Sr., whose encouragement, love, understanding, and unending support made possible the completion of this goal. I also acknowledge the support of the National Institute of General Medical Sciences, NIH Grant #1 TO2 GM 05007-01 MARC and the National Institutes of Health-Minority Biomedical Sciences Program, NIH-MBS Grant RR-8006.

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CHAPTER I

INTRODUCTION

Several genera of Coelomycetes form conidia that are distinguished by the presence of some kind of setula or appendage on the spore. One such genus is the recently established Pseudorobillarda. In this genus an uncertainty has prevailed on the origin, position, and nature of the setulae of the conidia. Consequently, this study was undertaken in order to shed further light on this problem.

It has been noted that there is a tendency in the taxonomy of coelomycetous members of the Fungi Imperfecti to regard the morphology of fructifications and conidia as providing characters secondary to the type of conidium ontogeny (Pirozynski and Shoemaker, 1971). To a considerable extent this practice prevails because the pattern of conidiogenesis has been evaluated in relatively few genera of Coelomycetes. Consequently, as stressed by Sutton (1971), there is a need for more detailed studies of pycnidial and pycnidioconidial development in members of the Sphaeropsidales.

There are no previously reported electron microscopic investigations on conidiogenesis in Pseudorobillarda or of other setulaceous-spored genera of coelomycetes. Studies at this level should resolve some of the questions on conidium ontogeny and concomitant setula formation in these fungi. This study has been undertaken, therefore, with the following objectives in mind:

(1) To correlate details on conidium ontogeny in this genus, as observed under light microscopy, with those found under electron

microscopy.

(2) To resolve the question of setula origin and position on conidia of Pseudorobillarda.

CHAPTER II

REVIEW OF LITERATURE

Pseudorobillarda phragmitis (Cunnell) Morelet, a Coelomycete, was originally isolated by Cunnell (1958) from a dead culm of Phragmitis communis Trin. submerged in a gravel pit, near Staines, Middlesex in England. Two years later Johnson and Hughes (1960) reported the occurrence of this fungus on panels of pine (Pinus spp.) and yellow poplar (Liriodendron tulipifera L.) that had been submerged in an estuary on the North Carolina coast. They also noted P. phragmitis on culms of Phragmitis communis that grew at the edge of a river emptying into the main course of the estuary.

Cunnell's (1958) studies and description of P. phragmitis were based mainly on cultures isolated from Phragmites stems and Carex leaves. He considered the fungus to be a member of the genus Robillarda and named it R. phragmitis. Since that time several investigators have studied this species, as well as other species placed in Robillarda, and have noted problems in the taxonomy of the genus (Frederick, 1962; Morelet, 1968; Kohlmeyer, 1969; Nag Raj et al., 1972). All of these investigators have commented on the presence of groups of species in the genus that were not congeneric. This has led ultimately to the establishment of a generic segregate in Robillarda to accommodate species with conidia of the R. phragmitis-type. This generic segregate has been given the name Pseudorobillarda (Morelet, 1968). As a consequence, Cunnell's (1958) R. phragmitis has been

transferred to Morelet's new genus Pseudorobillarda.

Pseudorobillarda phragmitis was described as having mature spores, which were uniseptate and fusiform, with thin, smooth, colorless walls, and a slight constriction at the median septum. Spores were also described as having several small oil droplets dispersed in the dense cytoplasm, giving the spore a pale olive-green color. One end of the spore was described as being rounded or obtuse and the other end more or less obtuse but bearing 2-4, usually 3, prominent appendages 15-23 µm in length. For convenience of terminology, the appendages will be referred to as setulae in the remainder of this paper.

The presence of 2-4 setulae on fusiform, typically uniseptate, colorless conidia has been a distinguishing feature of the genus Robillarda. Consequently, any pycnidial fungus with spores possessing these characters was usually placed in this genus. It is this feature, however, that has created the taxonomic confusion and has now resulted in the division of the genus.

In the published literature on the genus Robillarda the setulae are generally described as apical. For some species, however, it has not been clear as to whether they were borne on the basal or distal end of the spore. Sprague and Cooke (1939) stated that the setulae of their new species, R. agrostidis Sprague & Cooke, were borne at the tip of the spore. Sometime later, however, when Sprague (1951) described a second species, R. muhlenbergiae Sprague, he indicated that the setulae were basal and that those of the former species were probably similar. Sprague based his conclusions on the orientation of setulae on spores upon release from crushed pycnidia.

Confusion concerning the position of the setulae stemmed primarily from the description given by Cunnell (1958) and the comment by Sprague (1951) relative to their origin. Cunnell (1958) stated that the young spore first appears as a rounded projection from the center of the wall cell and, with increase in size, its cytoplasmic connection with the basal cell becomes attentuated. With further enlargement the outer wall was reported to become stretched and more difficult to see at the sides and distal end of the developing spore. Cunnell (1958) stated that the cytoplasmic connection with the basal cell is finally severed although the spore remains firmly attached by the thick outer wall at its proximal end. He did not clearly indicate the endogenous nature of the conidia in his observations.

Cunnell (1958) further stated that it is only after detachment of the spore that the setulae can be seen. He suggested that they might have formed from strips of the outer wall which curve away from the sides of the spore coat soon after detachment, leaving an apical cap which soon disappears. He postulated that the proximal parts of the setulae were apparently organized within the basal transparent region where they are bent back to join each other and the end of the spore. He further postulated that setulae straighten out when the transparent proximal cap is pushed off.

Saccardo (1884) described the genus Robillarda Sacc. as one characterized by fusoid, uniseptate, subhyaline, holoblastic conidia, ornamented with three hyaline, apical setulae, borne in ostiolate pycnidia. His description was an early but not the original characterization of the genus. Subsequently, it can only be speculated that the

species described by Saccardo formed conidia and setulae in a fashion different from the manner noted in Pseudorobillarda. It is on account of these differences that the genus Pseudorobillarda was separated from Robillarda. According to Nicot and Rouch (1965), type material of Saccardo's species are not available.

In a preliminary report on his observations on the genus Robillarda, Frederick (1962) reported that in some species setulae formation results from protrusive outgrowths at the apical end of the spore. He noted that in other species setulae were not the result of apical protrusions. Instead, they appeared to represent recurved strips of the wall of the conidiophore in which they formed. As a result of the differences in setulae formation, in combination with other concomitant morphologic variations, Frederick concluded that certain species presently assigned to Robillarda were not congeneric.

A few years later Breton and Faurel (1967) studied R. phragmitis and R. muhlenbergiae and suggested that they be reclassified since sporogenesis was so distinct in the two. Nicot and Rouch (1965) had come to a similar conclusion earlier and stated that two different types of organisms had been described under the name of Robillarda. They indicated that the first group comprised species with carbonaceous pycnidia, without well-marked ostioles, and with bicellular spores adorned with three apical bristles. This type was considered to conform to the Saccardian idea of the genus Robillarda. The second group was comprised of those species with lighter-colored, distinctly ostiolated pycnidia, with conidia-bearing setulae at the base, such as P. phragmitis and R. muhlenbergiae. Additional features mentioned as

distinguishing the two groups were that in the first, paraphyses are lacking and the mode of conidium ontogeny is holoblastic, while in the second group, paraphyses are present and conidium ontogeny is enteroblastic. On the basis of these differences the two groups were separated at the generic level. Morelet (1968) and Nag Raj et al. (1972) recognized these differences and established the generic name Pseudorobillarda to accommodate R. phragmitis and R. muhlenbergiae. Several genera of Coelomycetes, e.g., Neottiospora, Discosia, Kellermania, Bartalinia, Pestalotia, Pestalozziella, possess conidia with appendages, now termed setulae, such as P. phragmitis. However, the very brief generic descriptions and the stress placed on the setulate nature of the conidia have caused confusion in the taxonomy of many Coelomycetes.

Actual knowledge to date about comparative conidium ontogeny in the Coelomycetes has resulted primarily from light microscopic observations (Sutton and Sellar, 1966; Breton and Faurel, 1967; Subramanian and Ramakrishnan, 1953; Pirozynski and Shoemaker, 1971; Morgan-Jones, 1971; Morgan-Jones et al., 1972; Cunnell, 1957, 1958; Sprague, 1951; Nicot and Rouch, 1965). Often, however, light microscopic observations have been difficult and not fully reliable since the size of some morphological components, such as the setulae, often approach the limits of resolution of the light microscope.

Even though the importance of electron microscopy for resolving details of conidium ontogeny is obvious, there are as yet very few electron microscopic studies of conidiogenesis in the Coelomycetes.

Using light microscopy, White (1967) has reported on conidium

ontogeny in the genus Dinemasporium. He stated that the spores are murogenous in origin, developing as an expansion of the conidiophore tip. A constriction was reported to delimit the conidium from the conidiophore before it is released. Spore setulae did not appear to represent outgrowths from the ends or sides of the spore wall. In a few examples, however, refractive spots were noted near the end of young conidia. White stated that these spots were probably related to setulae development. No determination was made, however, on the origin of conidial setulae. He further stated that the majority of species appeared not to have setulae present on the intact conidium prior to its release from the conidiophore.

McVey and Gerdemann (1960) studied the setulaceous spored coelomycete Leptodiscus terrestris and reported that the spores first appear as small protuberances on the inner edge of the sporogenous cells. These protuberances were noted to enlarge and elongate and, as development continued, they appeared to be attached to the sporogenous cell by a short stalk. This stalk was noted to disappear when the spores matured. When the spores reached their mature size, the setulae formed. They reported that the setula on the upper end of the spore developed at the point of greatest curvature toward the concave side, and the setula on the lower end developed at the edge of the slightly flattened surface on the concave side of the spore.

In Kellermania, according to Sutton (1968), the setulae are "non-living deviations from the cell walls." He reported that the setula represents an extension of a persistent, non-gelatinizing sheath that envelops the entire conidium throughout its development.

According to Sutton (1968), the setula in Kellermania may be equivalent to the setulae in many other imperfect fungi, e.g. Dinemasporium, Menispora, etc. Pirozynski and Shoemaker (1971) stated that the setulae of P. phragmitis are similar to those in Kellermania in that they form from a persistent outer wall which, however, splits longitudinally with a recurving of the strips. This horizontal or longitudinal splitting of the outer sheath was reported to be accompanied by various degrees of gelatinization.

The above method of setula ontogeny is considered to be common to Neottiospora Desm. The species of Neottiospora, N. caricina (Desm.) Höhn., produces, in discrete immersed pycnidia, one-celled conidia with mucilaginous setulae. Cunnell (1957) clearly demonstrated that the setula in N. caricina is basal. This was later confirmed by Shoemaker (1965). Pirozynski and Shoemaker (1971) stated that the sheath around the developing conidium splits at the apex of the spore and is everted to form a funnel-shaped tail-like setula which may eventually tear into several irregular strands.

Subramanian and Ramakrishnan (1956) examined the fungus Neottiospora coprophila Speg. and decided that this species should be transferred to the genus Robillarda. They considered the nature of the setulae on spores of this fungus to be markedly different from those typical of spores of species of Neottiospora. N. coprophila is characterized essentially by having spores which are tapered and carrot-shaped, with one septum dividing the spore into unequal cells with apical setulae.

Even though it has been shown that some fungi are grouped together

because their conidia possess setulae, while differences in morphology of the conidia, and even of the setulae themselves, are ignored, it should be noted that the mode of origin of the setulae and their position in relation to the conidiophore promises to be of greater taxonomic value when their origin and structure are better understood.

CHAPTER III

MATERIALS AND METHODS

A culture of P. phragmitis was obtained from the Centraalbureau Voor Schimmelcultures, Baarn (Nederland). This culture was maintained in a 26 C Environ Room on freshly prepared oatmeal agar and Difco potato dextrose agar (PDA) in petri dishes or 3 oz. culture bottles.

Cultures utilized in light and electron microscope studies were allowed to grow from 30-45 days after subculture. Within this period of time immersed pycnidia developed in the culture media. Pycnidial maturation was evidenced by the presence of dark-brown pigmentation in the pycnidial wall and the discharge of spores.

Procedures for Light Microscopy

Water mounts of the spore material were made by crushing a pycnidium in a drop of deionized water. A drop of 25% glycerol was subsequently added to prevent drying. Bright-field studies of conidial development were observed through a Wild M-20 compound microscope equipped with phase contrast accessories and a Photo-Automat system for photography. Spore material was mounted in phloxine, ruthenium red, lactophenol and cotton blue, or Giemsa, for bright-field microscopy. Of the above stains, phloxine proved to be the most effective.

Phase contrast studies of conidial development were observed through a Phase Star microscope (American Optical Company) equipped with a Photo-Automat for photography. Photographs of developmental stages were taken on Polaroid type 105 P/N film.

Procedures for Scanning Electron Microscopy

For scanning electron microscopy (SEM) pycnidia were prepared as follows: whole pycnidia were placed in 3% glutaraldehyde prepared in a 0.1 M phosphate buffer, at pH 7.2, for 1 hr, rinsed for 3 hr in the buffer, dehydrated in 70%, 95%, 100% ethyl alcohol at 15 min intervals, infiltrated with two changes, at 15 min intervals, of a 1:1 ethyl alcohol and amyl acetate, and two changes, at 15 min intervals, of pure amyl acetate.

Material was also prepared for SEM using a technique of Panessa and Gennaro (1972). In this procedure the material was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, at pH 7.2, for 2 hr and rinsed for 2 hr in the buffer and placed directly in 2% uranyl acetate for 1 hr. The material was then rinsed in 3 changes of 10% glycerol and water and infiltrated with continuous graduations of glycerol through 50% for at least 4 days.

Materials fixed as described above, were studied in the Electron Microscopy Laboratory at the University of Georgia, Athens, Georgia. These materials were prepared for scanning electron microscopy (SEM) by placing them on nucleopore filters, that were affixed to double-stick tape (3M Co., Scotch Brand) mounted on clean specimen stubs. The mounted material was crushed and spread on the filter for critical point drying. After the drying process the specimens were sputter coated with palladium-gold (40:60) in a Hummer (Varian, Model VE 10). Specimens were observed in a Mark 2A Cambridge Stereoscan Electron Microscope at an angle of 45^{o}, using an accelerating voltage of 30 KV. Photographs of spore images were taken on Polaroid type 105 P/N film.

Procedures for Transmission Electron Microscopy

Material was prepared for transmission electron microscopy by first mincing pycnidia in a primary fixative. Several fixation procedures were initially attempted. The primary fixatives used were: 2.5% glutaraldehyde (Cole and Aldrich, 1971a), 3% glutaraldehyde (Hammill, 1972), 0.8% glutaraldehyde (Hammill, 1974), 4% paraformaldehyde (Hammill, 1971), and 4% paraformaldehyde-glutaraldehyde (Karnovsky, 1965).

When using the glutaraldehyde fixative, the material was fixed for 2-6 hr, rinsed for 2-3 hr in a 0.1 M phosphate buffer, and postfixed in 1% or 2% osmic acid at room temperature. The material was then rinsed for 2-3 hr and dehydrated. In the paraformaldehyde procedure (Pease, 1964), the material was aspirated for 5-10 min, and fixed overnight (ca. 15 hr) in the cold room. Fixed tissue was rinsed in 2-3 changes of deionized water for 30 min and secondary fixation followed in 0.6% or 1% unbuffered $KMnO_4$ for 1-2 hr at room temperature. Fixed material was rinsed as before (30 min) and dehydrated.

All material, regardless of fixation procedure, was dehydrated in an ethanol series that consisted of: 10 min each in 30-50-70 and 80% ethanol, 10 min each in 2 changes of 95% ethanol, and 3 changes of absolute ethanol (20 min each). The material was subsequently carried through 2 changes (30 min each) of propylene oxide for final dehydration.

Following dehydration, the material was infiltrated by placing the tissue in a 3:1, 1:1, 1:3 ratio of propylene oxide and the embedding mixture. Spurr's (1969) embedding medium was used. The medium was

added to the vial containing the specimens by increasing the concentration of Spurr's to about 1:1, Spurr's propylene oxide mixture, in a 3- or 4-step process. The volume was increased to about 1/3, mixed, and allowed to stand for approximately 15 min. This step was repeated until a 50:50 mixture was obtained. The small test tube holding the mixture was then placed in a larger test tube on a roller drum and permitted to infiltrate overnight. Afterwards, the embedding mixture was replaced with fresh full-strength Spurr's and returned to the roller drum for a 6-7 hr infiltration. Following infiltration, specimens were embedded for sectioning.

Sections were cut with a diamond knife on a LKB III 8800 ultramicrotome and were mounted on 200 mesh copper grids, coated with formvar. Mounted sections were post-stained for 5-10 min in lead citrate (Reynolds, 1963). Most sections were double-stained using DTMO-dipotassium tetramethyl osmate (Hinckley and Murphy, 1975). Ruthenium red (Springer and Roth, 1973) was used to stain the mucilaginous matrix. Sections were examined in a RCA EMU-4 electron microscope at 50 KV.

When fixation techniques were compared, the paraformaldehyde-potassium permanganate fixation and the lead citrate-DTMO staining gave the best results. Consequently, all observations reported here are based on material fixed and stained according to the above procedures.

All micrographs were taken on 3-1/4" X 4" Kodak Electron Image Plates and developed in Dektol. Photographic images were enlarged on a Durst S-45 EM Enlarger and printed on Kodabromide paper.

CHAPTER IV

OBSERVATIONS

Cultural Characteristics of P. phragmitis

The growth pattern of P. phragmitis varied with the culture medium. On freshly prepared oatmeal agar, mycelial growth was minimal and pycnidial formation was abundant (Fig. 1). Pycnidial formation usually occurred after 3-4 weeks of growth on this medium. On PDA pycnidial production was heavy and mycelial growth was more restricted than that developing on oatmeal agar (Fig. 2). A period of 3-4 weeks was also required for pycnidia to form on these media. Growth on malt agar, as shown in Fig. 3, was principally mycelial with minimum pycnidial formation.

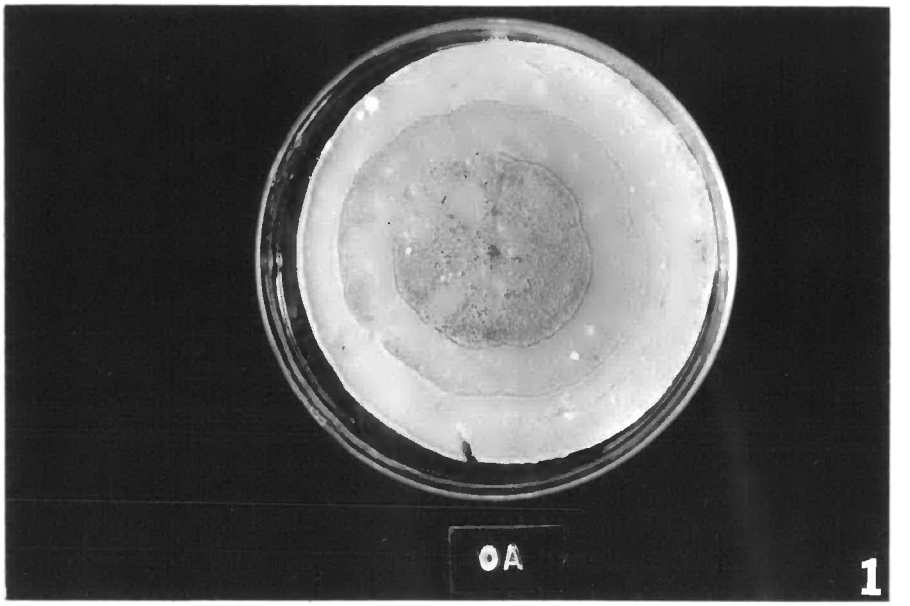
As shown in Fig. 4, pycnidia of P. phragmitis are conspicuously ostiolate. This is one of the features reported as distinguishing pycnidia of this genus from Robillarda. On the various culture media pycnidia occurred singly or in clusters, were light to dark brown in color, and were embedded in the medium. No obvious differences in conidium morphology were noted when cultures growing on different media were compared.

Light Microscopy

Stages in the development of P. phragmitis conidia were studied by the procedure previously described. It was noted that spores originate from short irregularly-shaped, thin-walled basal cells that form the innermost layer of the pycnidial wall. Paraphyses also arise

Fig. 1. Pycnidial development on freshly prepared oatmeal agar. Note concentric pattern and minimal mycelial growth.

Fig. 2. Pycnidial development on PDA agar showing mycelial growth.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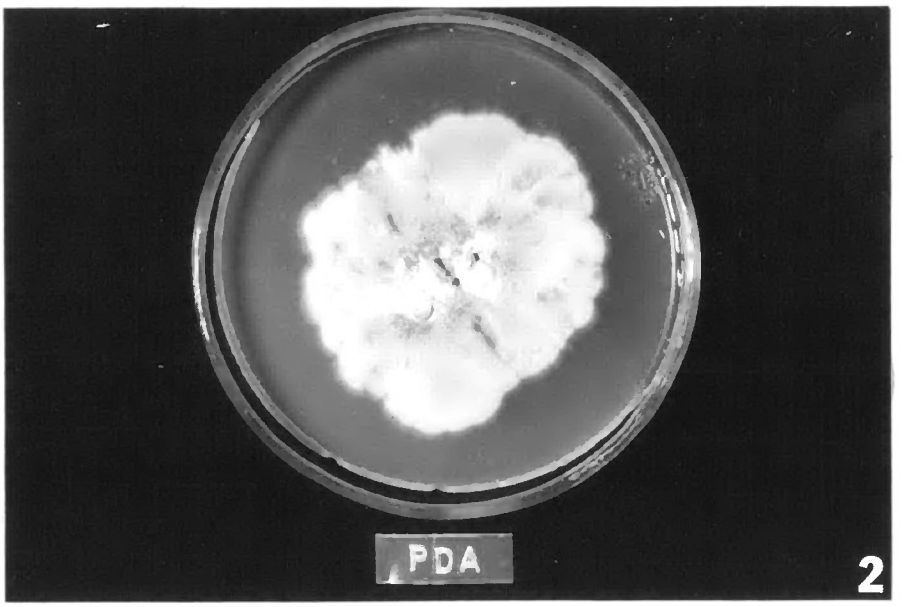
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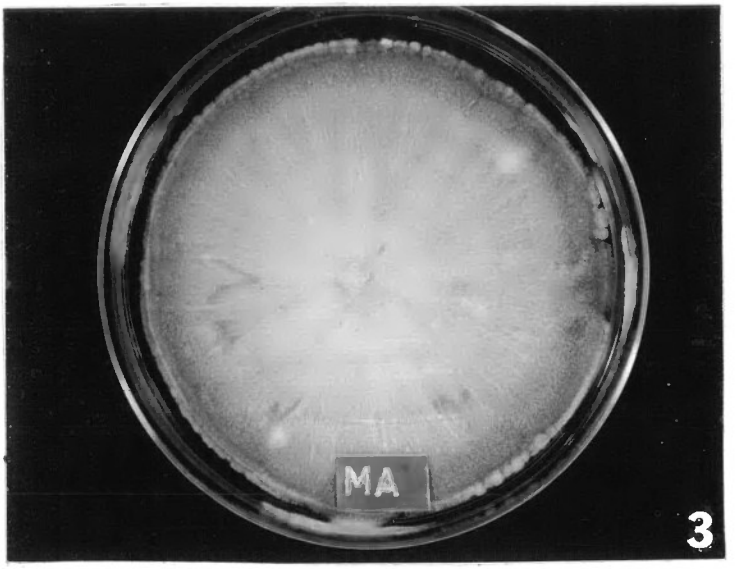
Fig. 3. Pycnidial development on malt agar. Note high mycelial growth.

Fig. 4. Pycnidial development on oatmeal agar. Note ostioles (arrow).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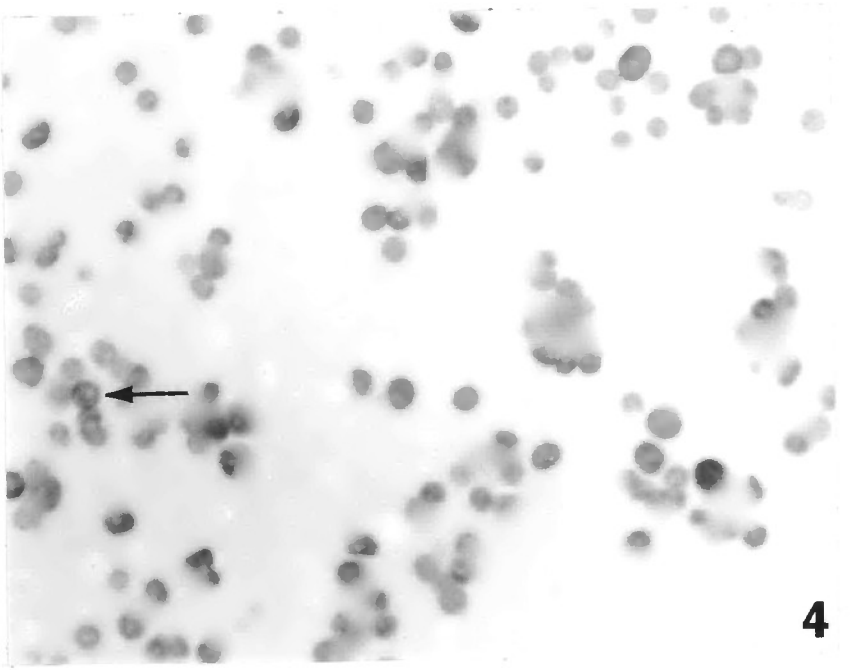
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from the same cells that give rise to conidia.

Conidium development begins when a basal cell, filled with dense cytoplasm as shown in Fig. 5, forms a bud-like protrusion which gradually swells. In Figs. 6-8 it can be noted that the bud, which is the initial of the conidiogenous cell, increases in length apically with little corresponding increase in width. As growth in length of the conidiogenous cell continues, the cytoplasmic connection between it and the basal cell gradually tapers (Fig. 9). At this stage the spore has not been delimited and retains cytoplasmic continuity with the protoplast in the basal cell from which it has developed. Subsequently, this connection becomes greatly constricted and finally breaks. The basal portion of the protoplast, that is to become the next conidium, rounds off and the new spore is delimited.

When continuity between the base of the protoplast of the developing conidium breaks with that of the basal cell, continuity is still maintained between the spore protoplast and its cell wall at its distal end. This connection is evident by the presence of a fine cytoplasmic strand, extending from the cell wall to the spore protoplast, and a refractive spot at the tip of the cell.

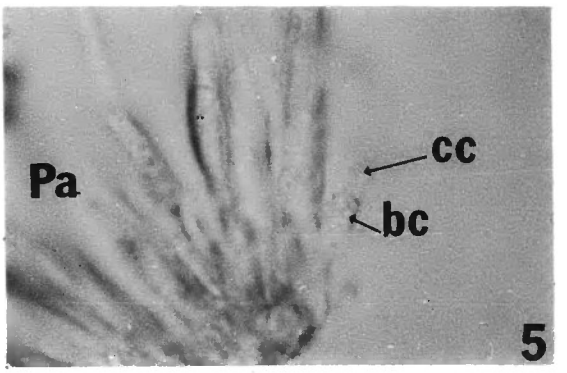
Following spore delimitation, as shown in Fig. 11, a wall appears around the protoplast that is differentiating into the conidium. This wall is distinct from the wall that originally surrounded the protoplast prior to its delimitation as a spore. The old wall becomes the wall of the conidiophore. At this stage the only place where there is a connection between the newly developed conidium and the original cell wall is at its apical end. The wall of the conidiophore is continuous,

Figs. 5-9. Early stages of conidiogenesis of *P. phragmitis*.

5. Nascent stage in development showing paraphysis and conidiophores. Note conidiogenous cell (cc) and basal cell (bc). X1250

6-8. Early stages in development showing increase in length of conidium initial. X1250

9. Later stages in conidiogenesis showing cytoplasmic connection between conidium initial and basal cell beginning to taper into a cytoplasmic constriction. X1250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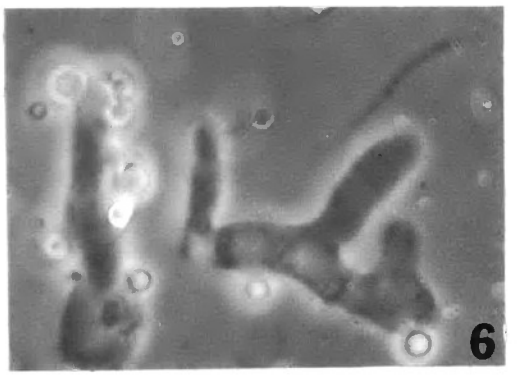
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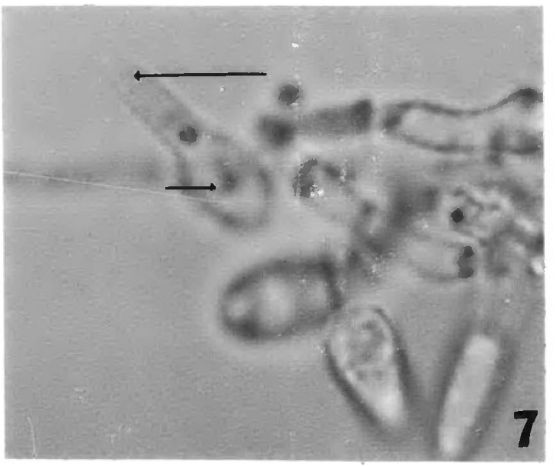
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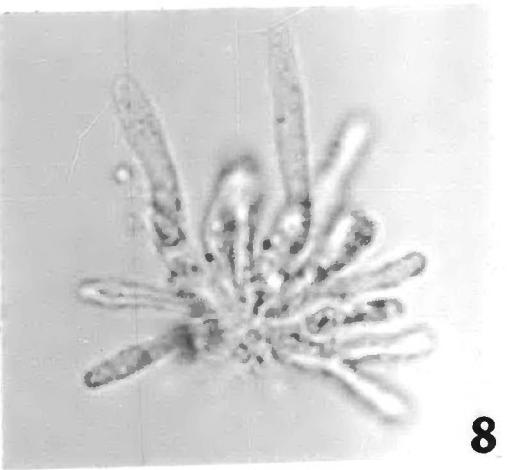
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Figs. 10-13. Later stages in conidiogenesis of *P. phragmitis*.

10. Stage in conidiogenesis showing separation of cytoplasmic connection at base of conidium initial and refractive spot at apical end. X1250
11. Stage in conidiogenesis showing a thin line extending from the tip of the spore to the conidiophore wall. X1250
12. Stage in conidiogenesis showing developing rounded basal end of developing conidium. Spore remains firmly attached at apical end to thick outer wall of the conidiophore. X1250
13. Detached spore with apical cap. X1250


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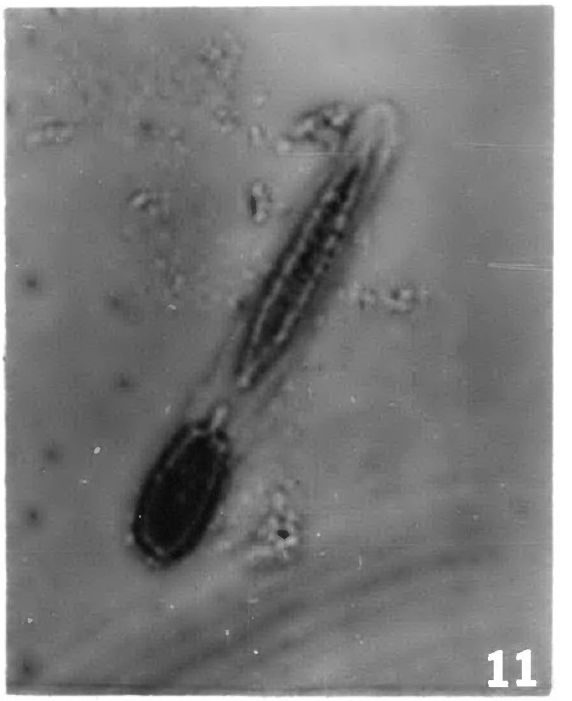
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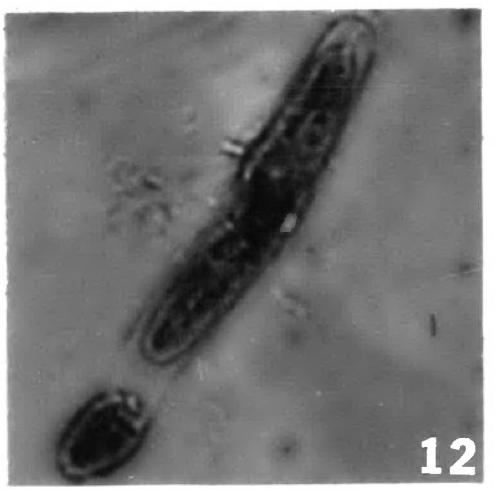
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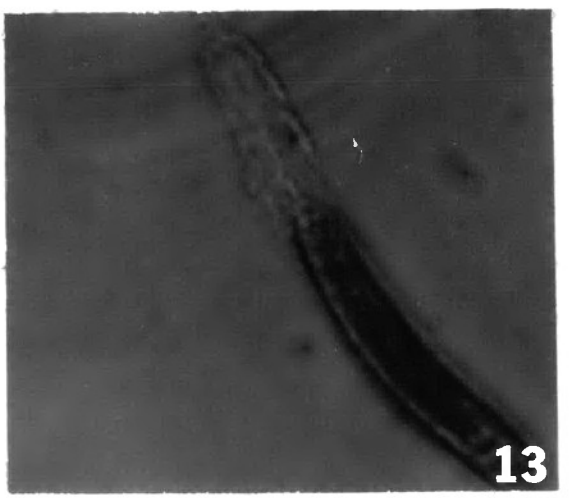

13

however, with the wall of the basal cell (Fig. 12). The spore is aseptate at this stage of development. Later on, either while still within the conidiophore or after its release, a median septum develops within the spore. Occasionally conidia become two- or three-septate.

The setulae can be seen only after the spore is released from the conidiophore. They are apically attached and appear to originate as strips from the conidiophore wall. The upper end of the conidium is commonly extended into a narrowed region that becomes a pedicel-like structure to which the setulae are attached (Figs. 13-16).

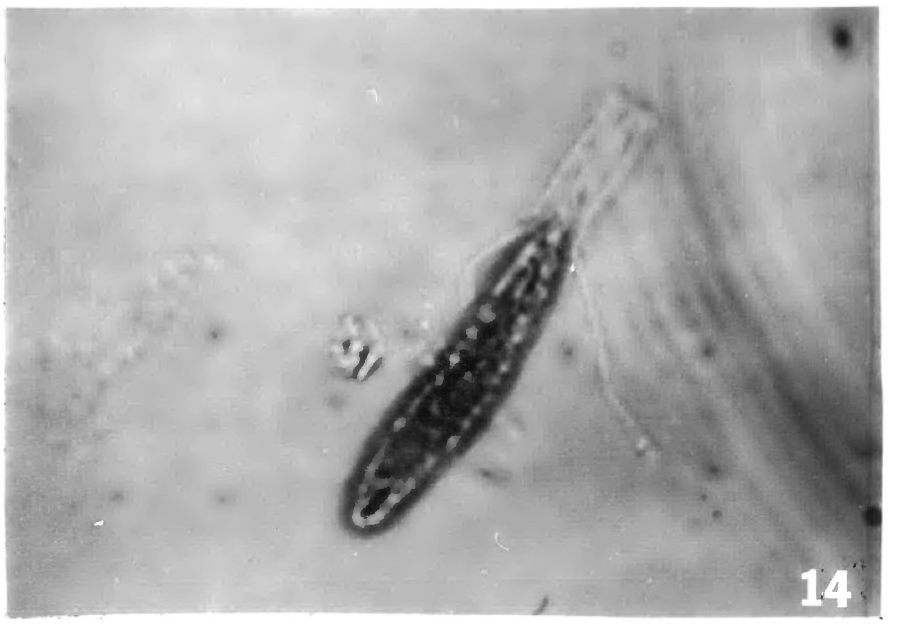
Fully developed conidia are shown in Figs. 17-19 after their release from conidiophores. Oil droplets and one or more septa are present in these spores. Setulae are also conspicuous. Following release of a conidium, remnants of the old conidiophore wall persist and a new conidiogenous cell develops within it. Proliferating basal cells are shown in Fig. 20. The development of successive spores follows the pattern previously described. Remains of a previous conidiophore wall, surrounding the base of a newly developing conidiogenous cell, are shown in Fig. 20. Several conidia are produced within the remnants of walls of preceding conidiophores.

Scanning Electron Microscopy

Some details on spore development and additional observations on setula form were obtained with the use of scanning electron microscopy (SEM). Pycnidial material was prepared for SEM observations by the methods previously described.

In Fig. 21 a clump of conidiophores is shown. The surface of the conidiophores at low magnification appears to be smooth. The wrinkled

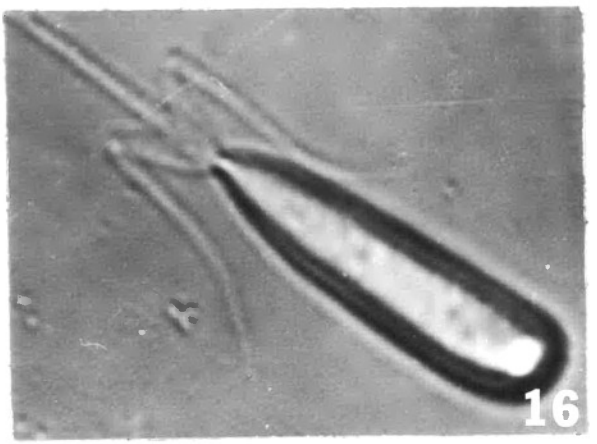
Figs. 14-15. Detached spores with apical cap and setulae. X1250


14


15

Figs. 16-20. Mature conidia and early stage in development of second proliferating conidium.

16. Spore with setulae associated with conidiophore wall. X1250
17. Mature spore with setulae and septum. X1250
18. Mature spore with pedicel, setulae, and conspicuous oil droplets. X1250
19. Multiseptate spore. X1250
20. Early stage in development of second proliferating conidium. Note the remnant of an old conidiophore wall (arrow). X1250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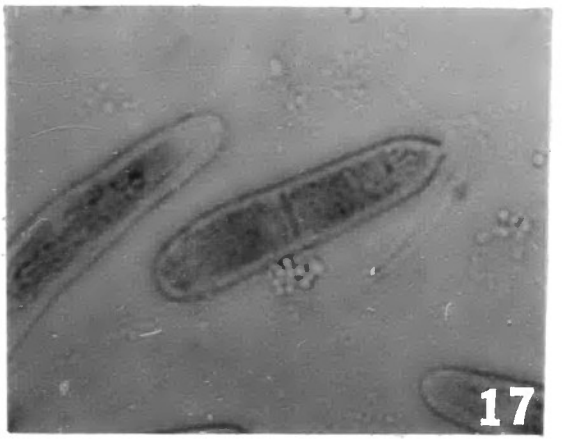
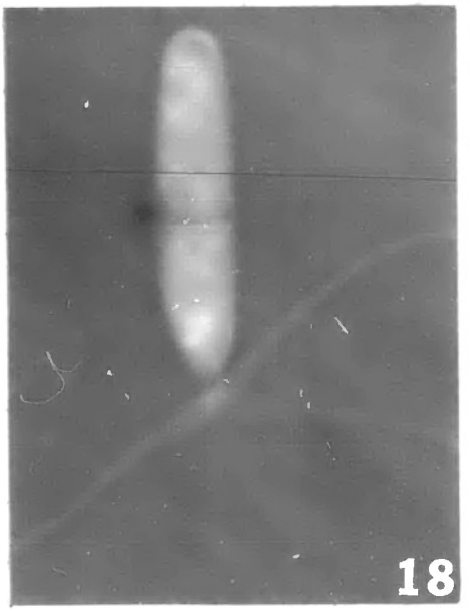

17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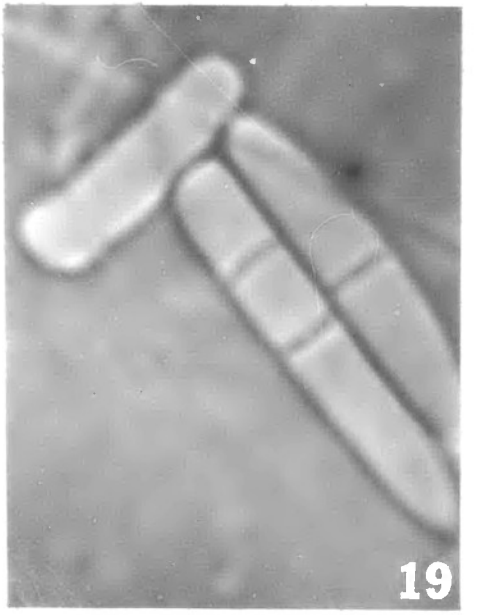
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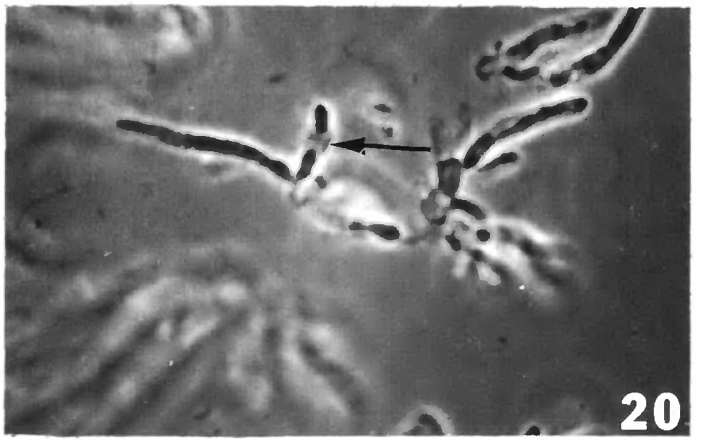
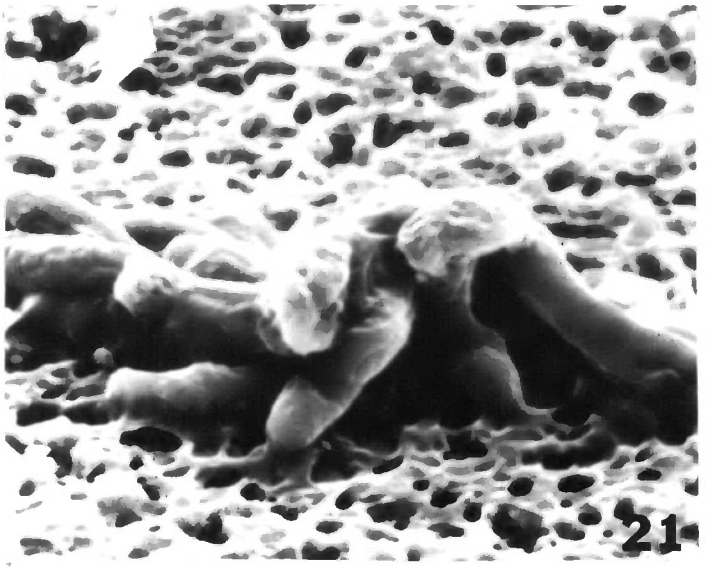

20

Figs. 21-23. SEM views of conidiophores, conidia, and paraphyses.

21. Clump of conidiophores. X4500

22. Conidia showing septa. Note paraphysis. X4500

23. Tip of paraphysis (Pa) and a conidium. X9000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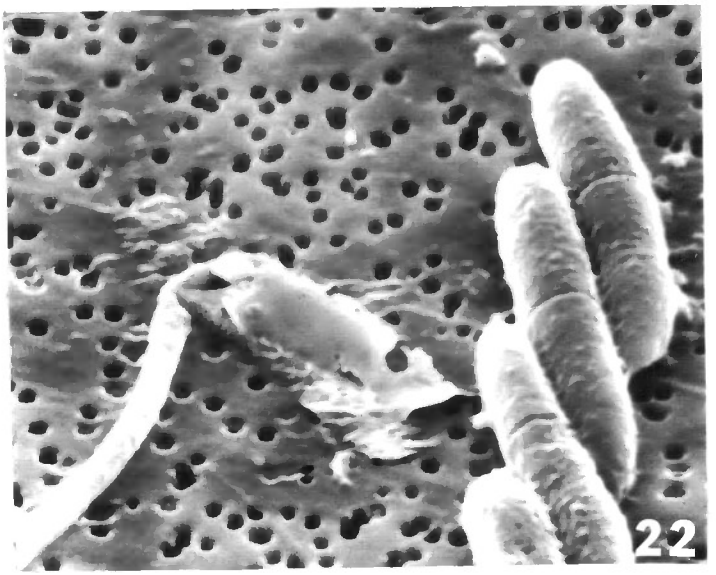
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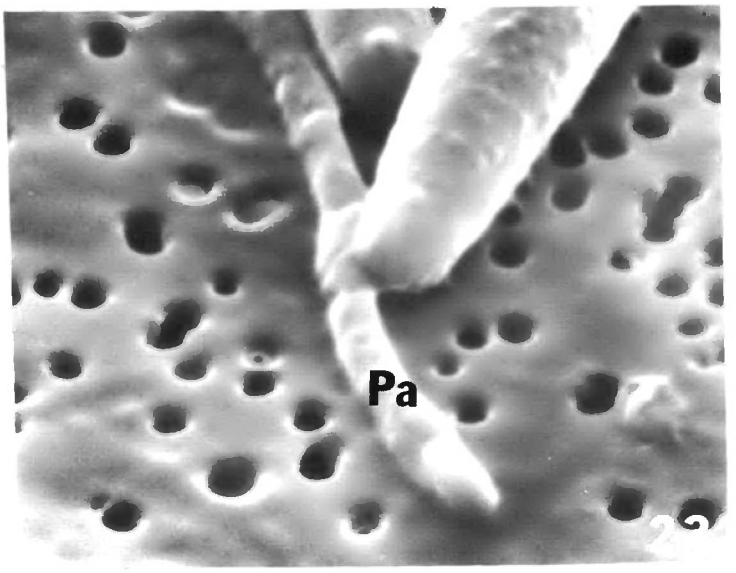

Pa

surface, apparent at high magnification, is probably an artifact due, no doubt, to glutaraldehyde fixation and dehydration. Less shrinking and distortion of pycnidial material occurred when glycerol substitution and critical point drying followed glutaraldehyde fixation.

Whole conidia and parts of paraphyses are shown in Figs. 22-24. A septum is conspicuous in most conidia and the pedicel-like apical projection that bears the setulae is evident in some. The slightly wrinkled surface of each spore is probably due to the surface deposit of the mucilaginous matrix that surrounds released spores within the pycnidium. In Figs. 22 and 24 spores appear clumped in a common matrix.

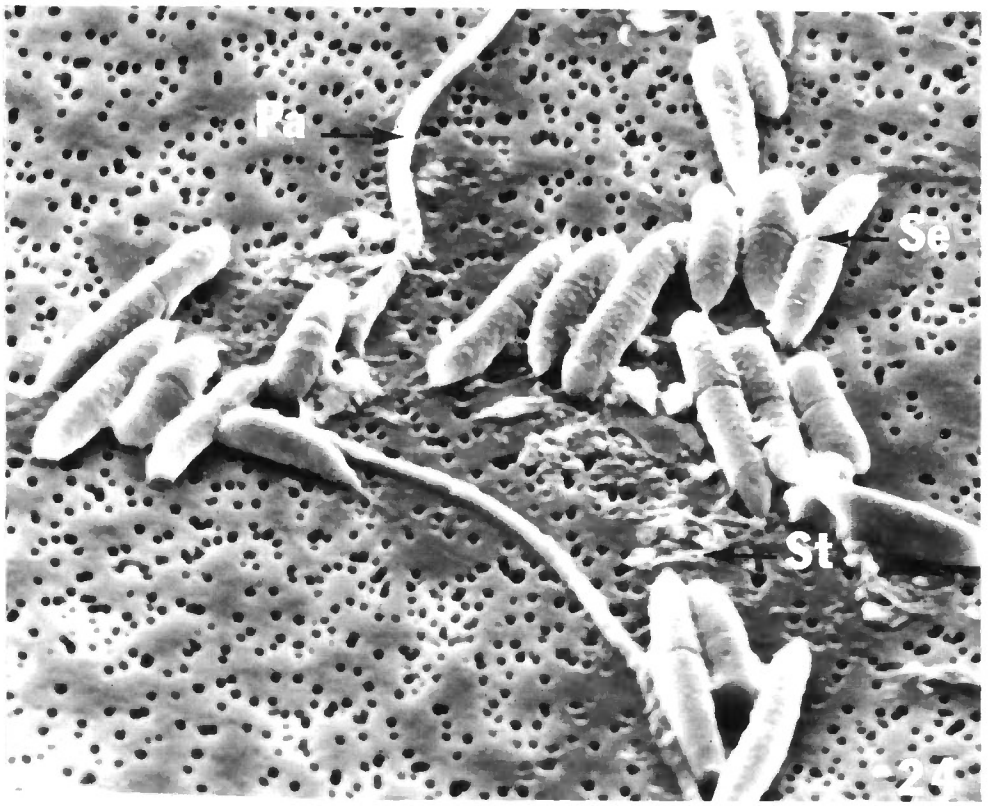
In Fig. 25 the apical end of a conidium is shown. The slender knob-like projection is a part of the spore that is referred to here as the pedicel. As indicated previously, the setulae are attached to this part of the spore. Light microscopy observations revealed that within this structure, during spore development, continuity of the cytoplasm between the spore protoplast and the conidiophore cell wall persists.

In Figs. 26-27 setulae are evident on some of the spores. The manner in which they appear to fuse with the matrix covering the spores suggests a flexible mucoid nature for these structures.

Transmission Electron Microscopy

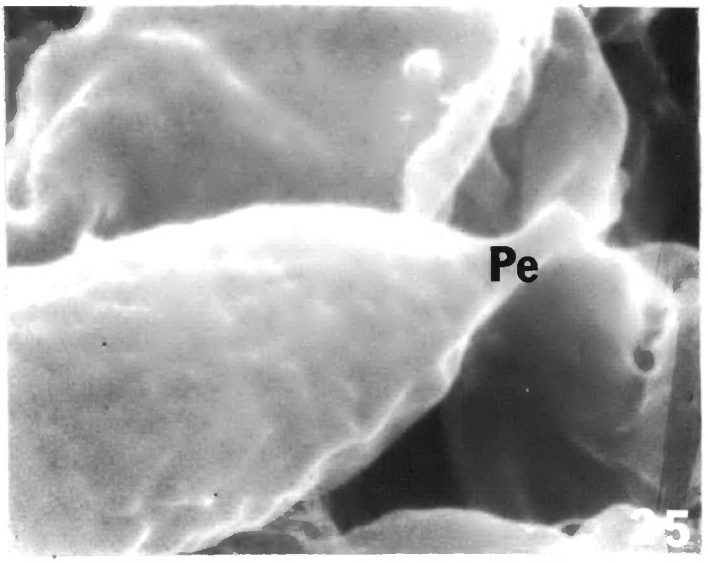
Critical events in conidium ontogeny in Pseudorobillarda have been observed, with the first evidence of conidiogenesis being the appearance of an outgrowth from the apical end of the basal cell (Fig. 28). The conidial initial and basal cell are clearly shown to be enclosed

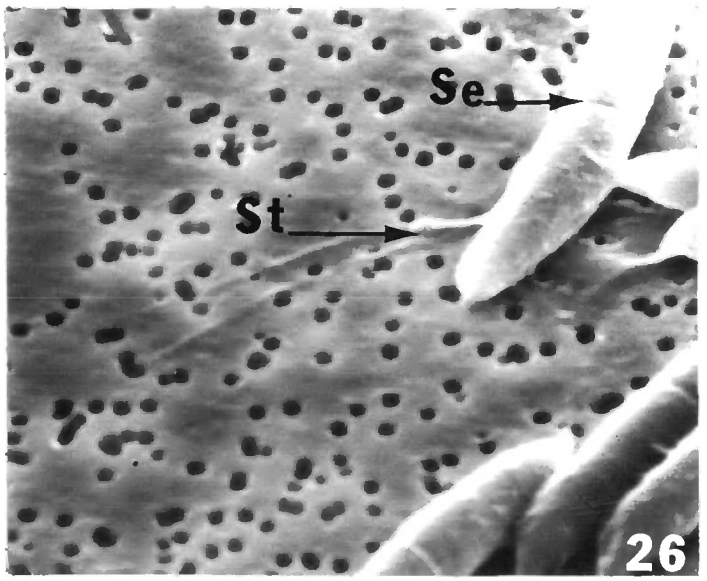
Fig. 24. SEM view of mature conidia showing septa (Se) and setulae (St). Note paraphysis (Pa). X4500

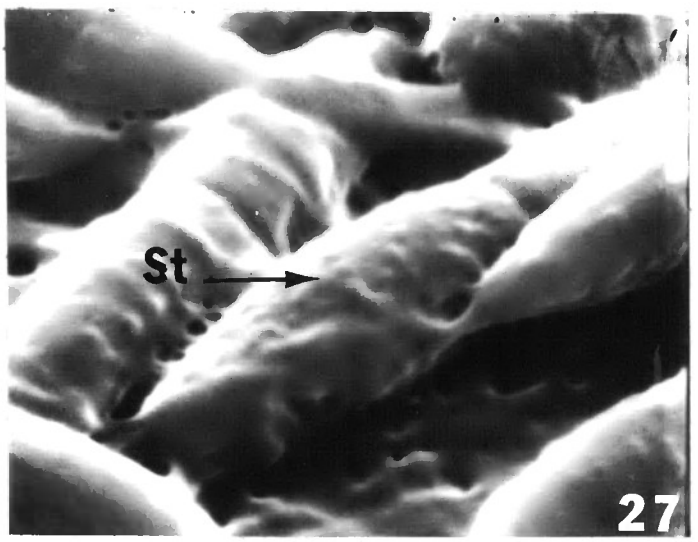

Se
St

Figs. 25-27. SEM views of conidia showing pedicel, septum, and setulae.

25. View of conidium showing pedicel (Pe). X22,000
26. View of conidium showing septum (Se) and setulae (St). X4500
27. Mature conidia showing setulae (St). X9000


Pe
5

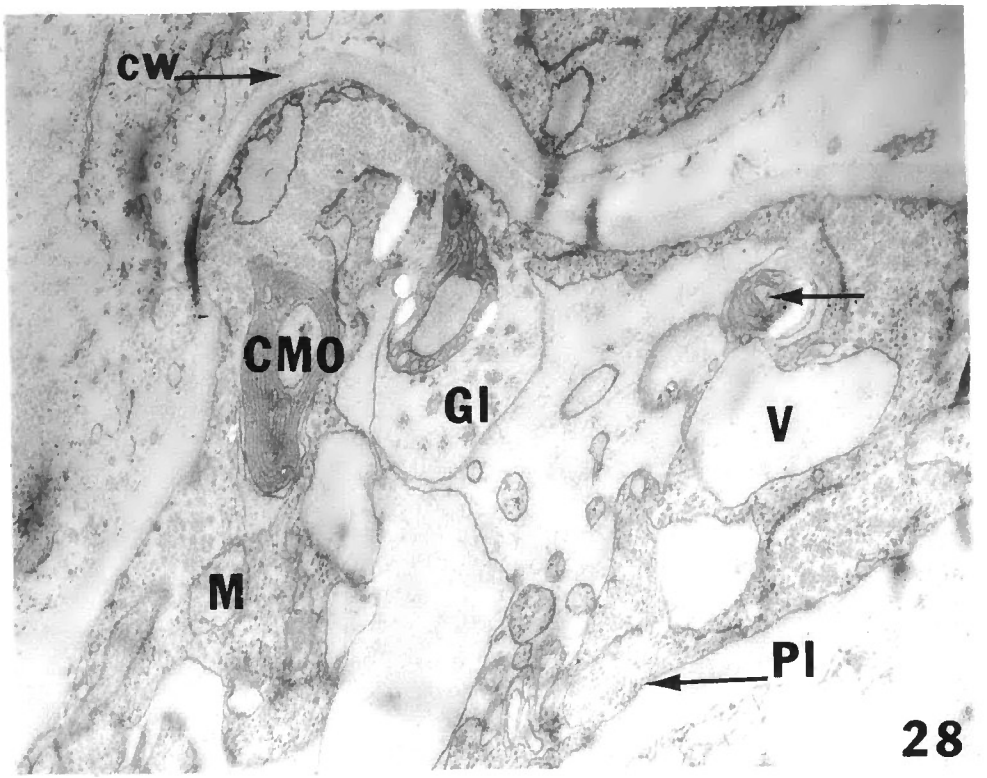

Se
St
26


St
27

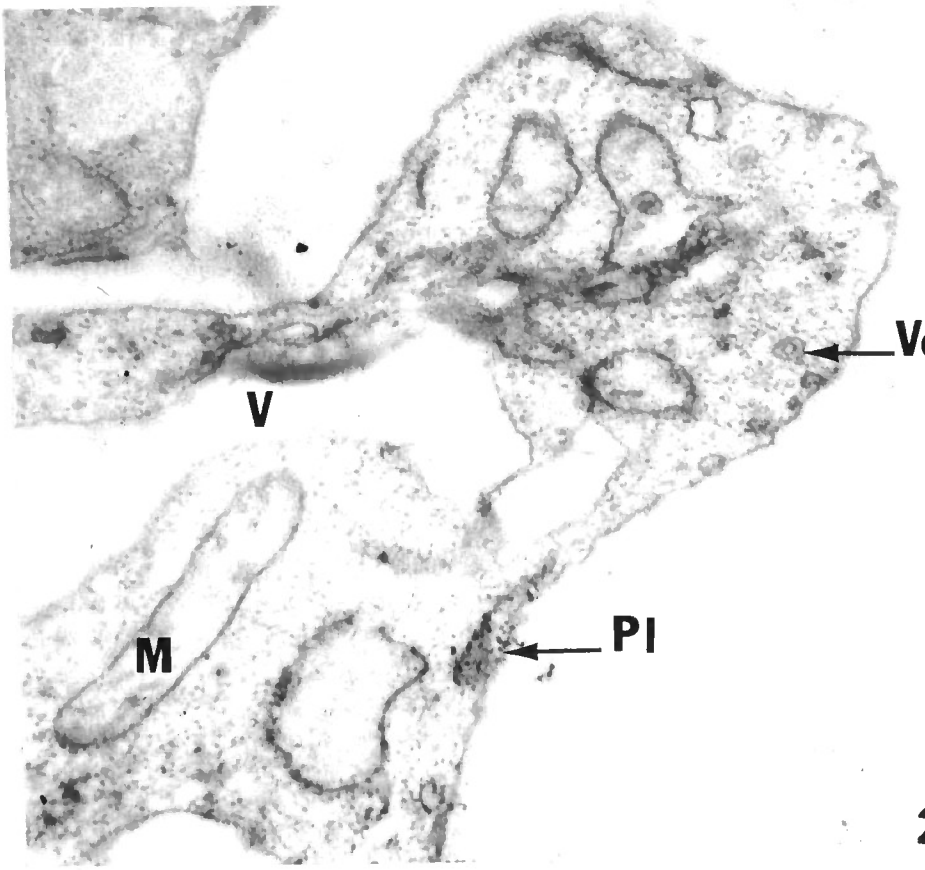
Figs. 28-29. TEM micrographs showing early stages in conidiogenesis.

28. Conidium initial developing from conidiogenous cell. Note mitochondria (M), vacuoles (V), concentric membrane organelles (CMO) - some with electron-dense inclusions (arrow), and common wall (cw) of conidium initial and conidiogenous cell. X33,000

29. Further development of conidium initial. Mitochondria (M), vesicles (Ve), vacuoles (V), and convoluted plasmalemma (Pl). X27,000



28



29

within a common wall (Fig. 28).

As the conidium initial elongates (Figs. 29-34), it remains within the common wall. Figs. 34-35 show also that associated with conidium elongation is a gradual tapering at the basal end of the developing spore. This tapering continues until the protoplast of the conidium initial has separated from the protoplast of the basal cell. The developing conidium remains enclosed within a single wall until it is fully delimited and is no longer attached to the basal cell protoplast. Following delimitation a new wall develops around the conidium. The old wall of the conidiophore persists outside of this wall. A remnant of an old conidiophore wall is shown in Fig. 35. This remnant plays no role in the development of succeeding conidial initials.

Fig. 36 shows a longitudinal view of a portion of an immature conidium. The conidium is aseptate at this stage of development. As development of the conidium progresses, however, a median septum forms. A profile of endoplasmic reticulum (ER) may be involved in the initial formation of the conidial septum (Figs. 40-41). In these figures a single ER profile is oriented across the newly delimited spore at the position where a septum normally forms. Further development of the septum is associated with the centripetal invagination of the plasma membrane (Fig. 37).

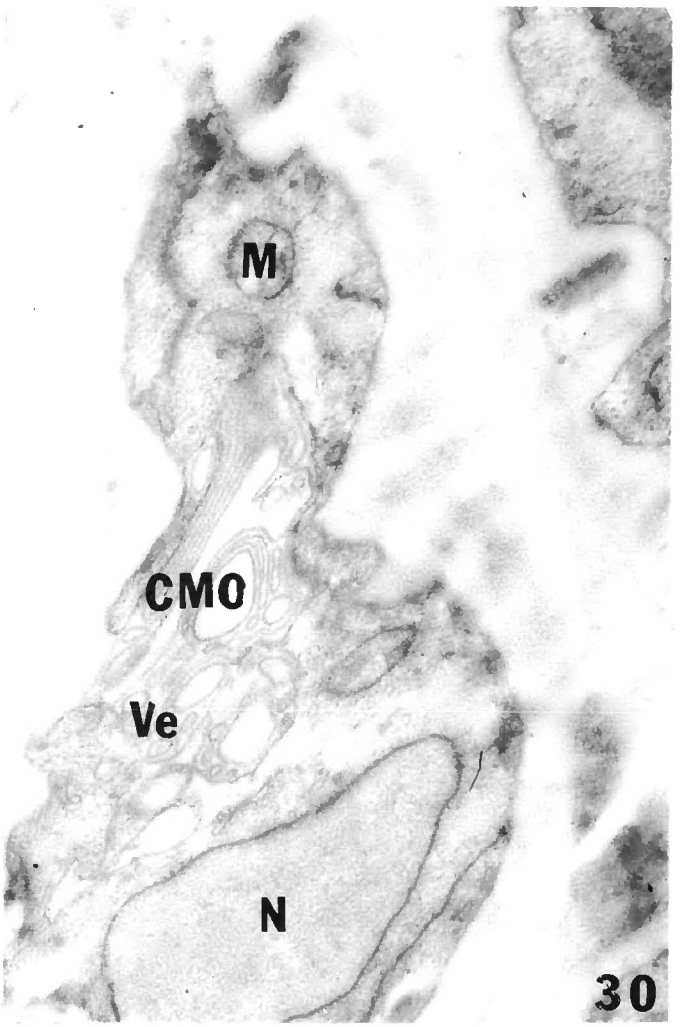
A later stage in septum formation is shown in Fig. 38. Following full septum formation a cytoplasmic connection persists between the protoplasts of the two cells. The walls deposited at the septum is continuous with the wall of the main body of the spore (Fig. 44).

A two-celled conidium is shown in Fig. 39. This micrograph shows

Figs. 30-31. TEM micrographs showing elongation of conidial initial.

30. Conidial initial showing concentric membrane organelles (CMO) fragmenting into vesicular elements. Note nucleus (N), mitochondria (M), vesicles (Ve). X39,200

31. Conidium initial elongating. Note the common wall (cw), vacuoles (V), nucleus (N), and mitochondria (M). X36,000


M
CMO
Ve
N
30

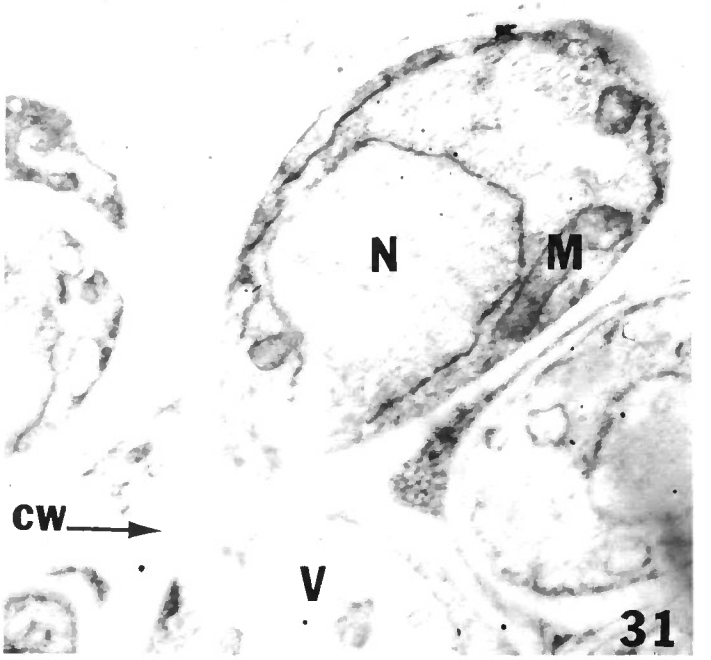
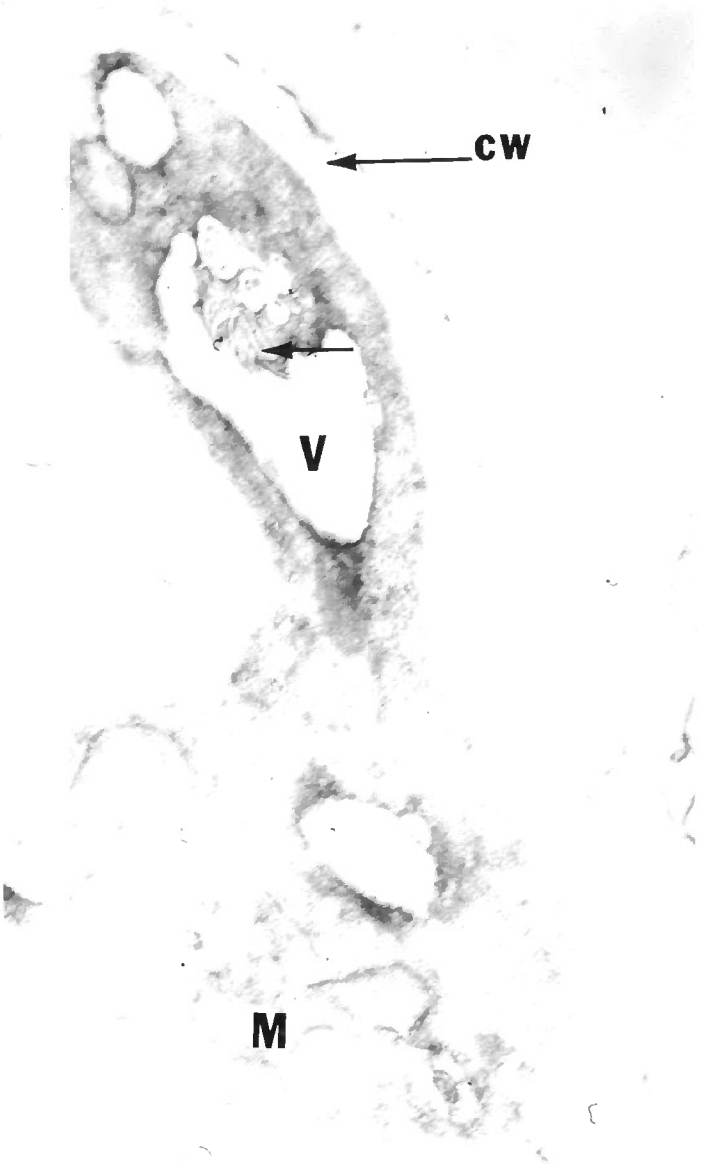

N
M
cw
V
31

Fig. 32. Further elongation of conidium initial. Note common wall (cw), mitochondria (M), and vacuole (V) with membranous material (arrow) indicating further fragmentation. X21,250

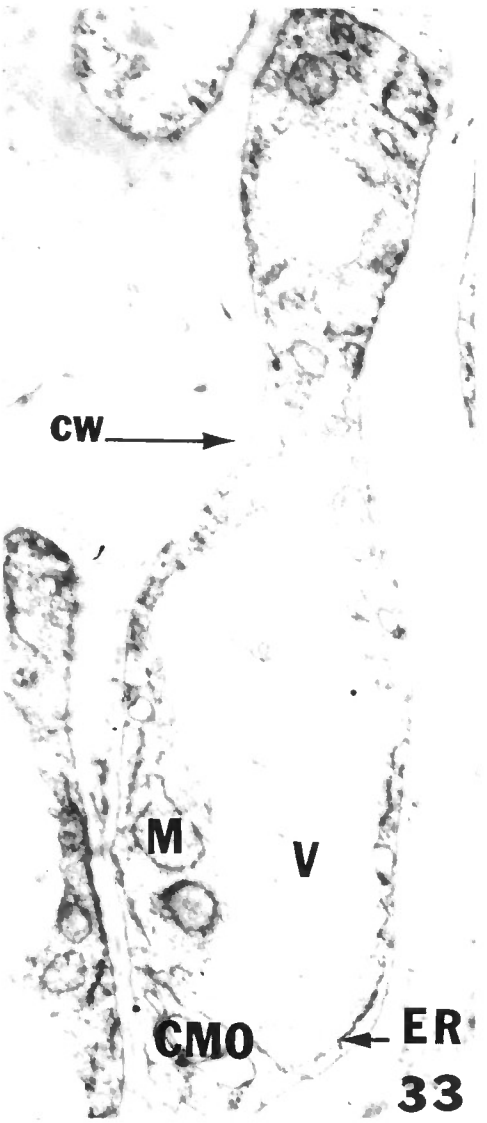


32

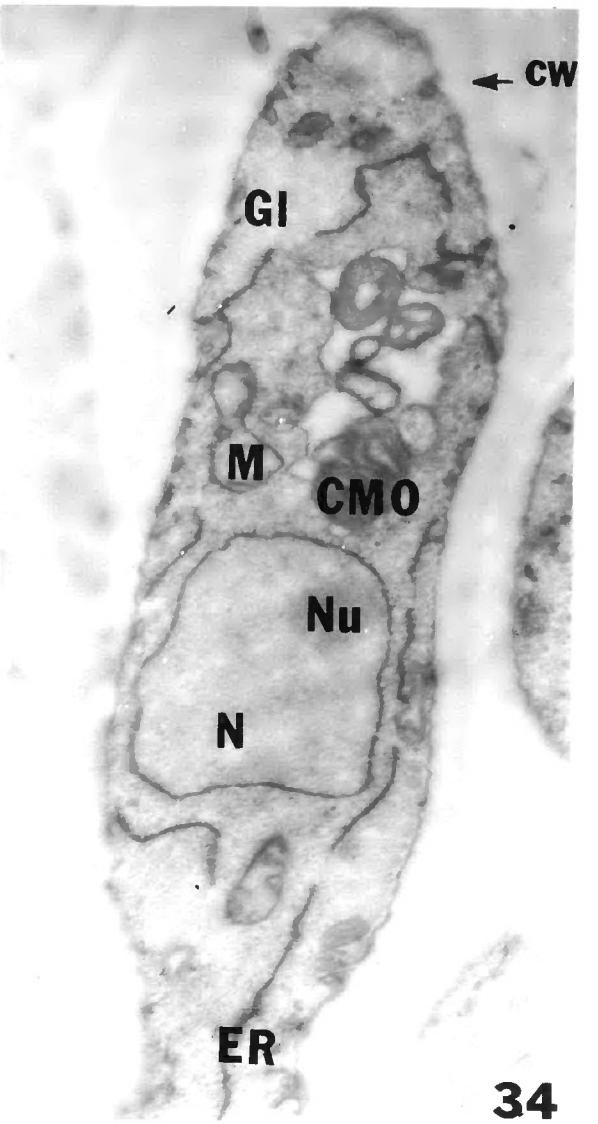
Figs. 33-34. TEM micrographs showing additional stages in conidial development.

33. Further elongation of conidium initial. Note segments of endoplasmic reticulum (ER), vacuole (V), mitochondria (M), membranous material (CMO), and the common wall (cw). X14,400

34. Conidiogenous cell beginning to taper at basal end. Note nucleus (N), endoplasmic reticulum (ER), glycogen (Gl), mitochondria (M), nucleolus (Nu), common wall (cw), and membranous material (CMO). X19,200



33



34

Fig. 35. Electron micrograph showing tapering of protoplast of conidial initial at basal end. Note membranous material (CMO), common wall (cw), glycogen (Gl), and a remnant of an old conidiophore wall (rcw). X34,000

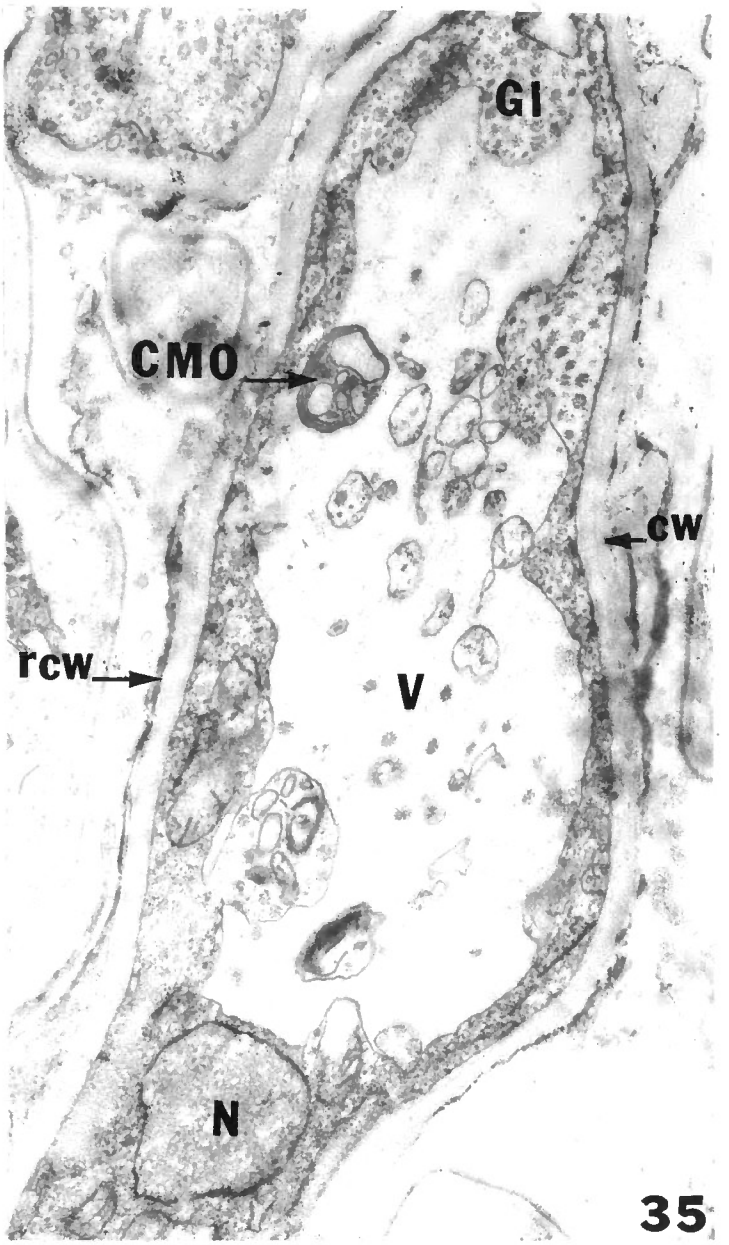
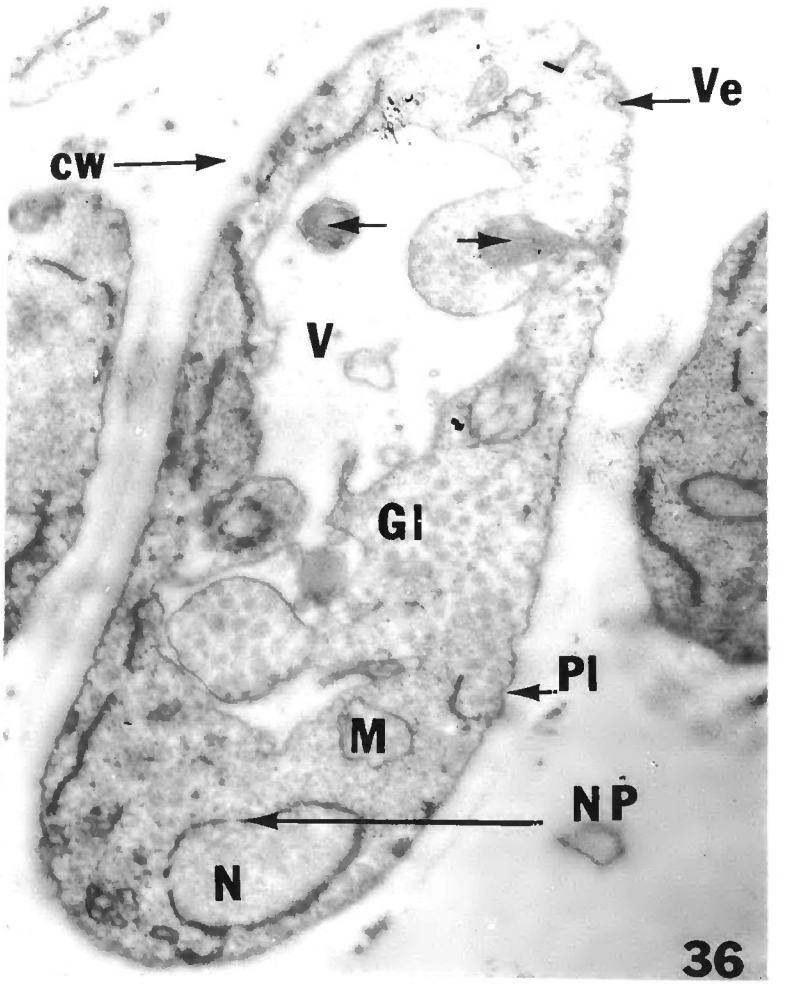

Gl
CMO
cw
rcw
V
N
35

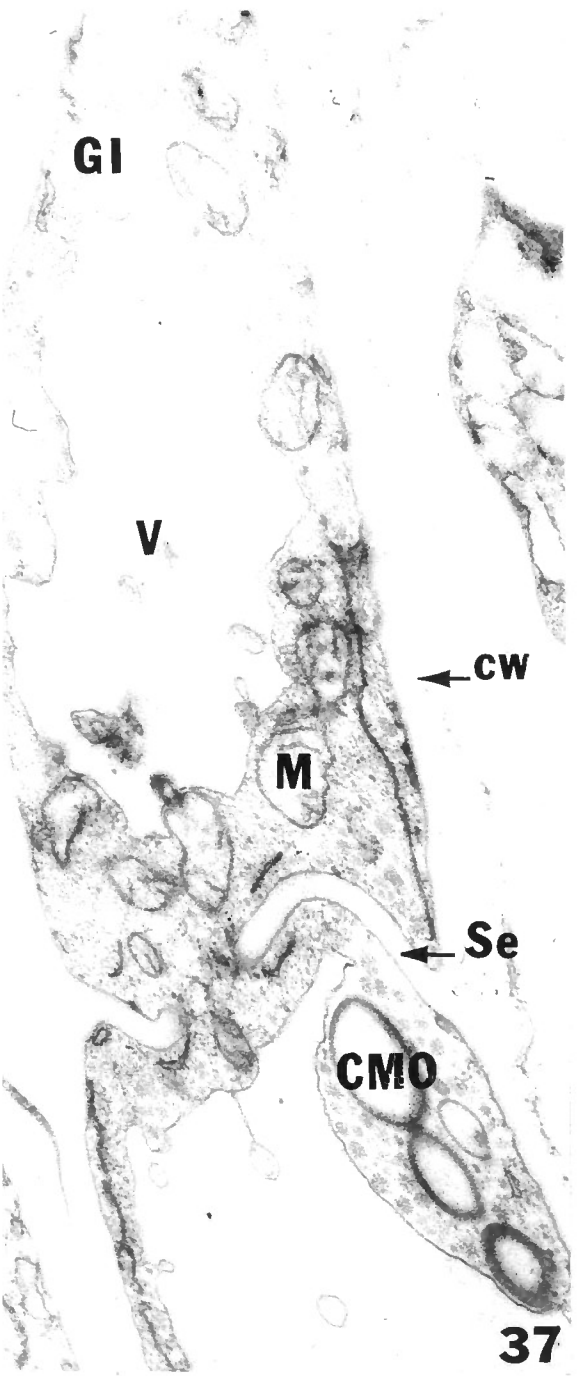
Fig. 36. Electron micrograph showing longitudinal view of delimited immature conidium. Note membranous material (arrows), vesicles (Ve), nucleus (N) with nuclear pores (NP), convoluted plasmalemma (Pl), and mitochondria (M), glycogen (Gl), and common wall (cw). X50,400


Ve
CW
V
GI
PI
M
NP
N
36

Figs. 37-38. TEM micrographs showing septum formation.

37. Septum formation in developing conidium. Note invagination of septum (Se), mitochondria (M), endoplasmic reticulum (ER), glycogen (Gl), vacuole (V), common wall (cw), and concentric membrane organelles (CMO). X22,000

38. Later stage in septum (Se) formation. Cytoplasmic connection persists between the protoplasts of the two cells. Note mitochondria (M), vacuole (V), and nucleus (N). X30,000


Gl
V
cw
M
Se
CMO
37

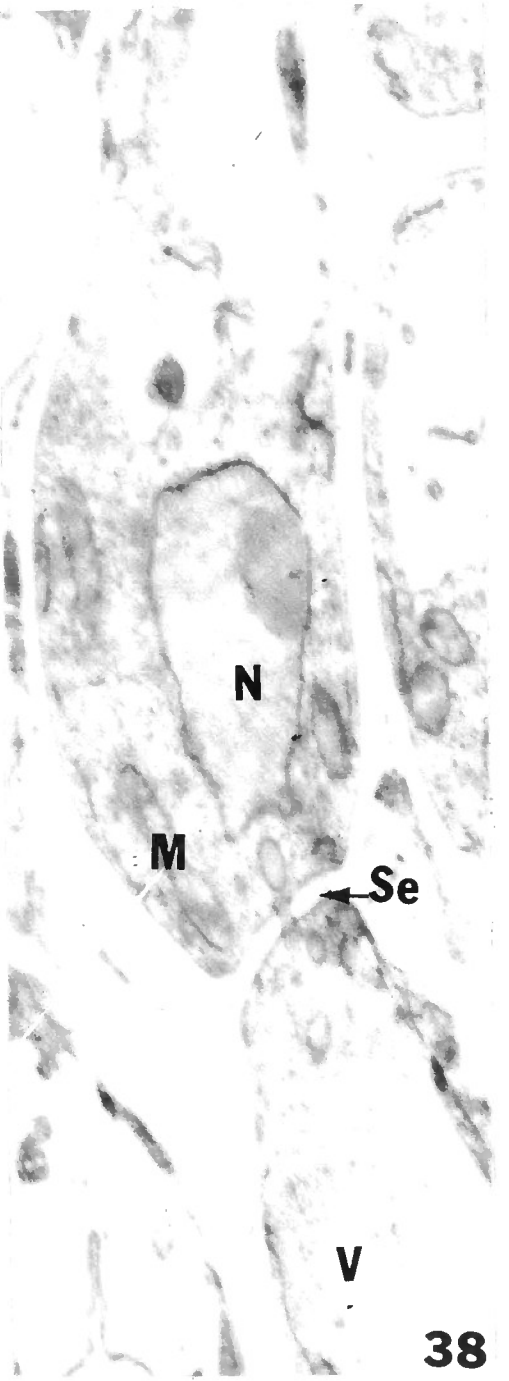
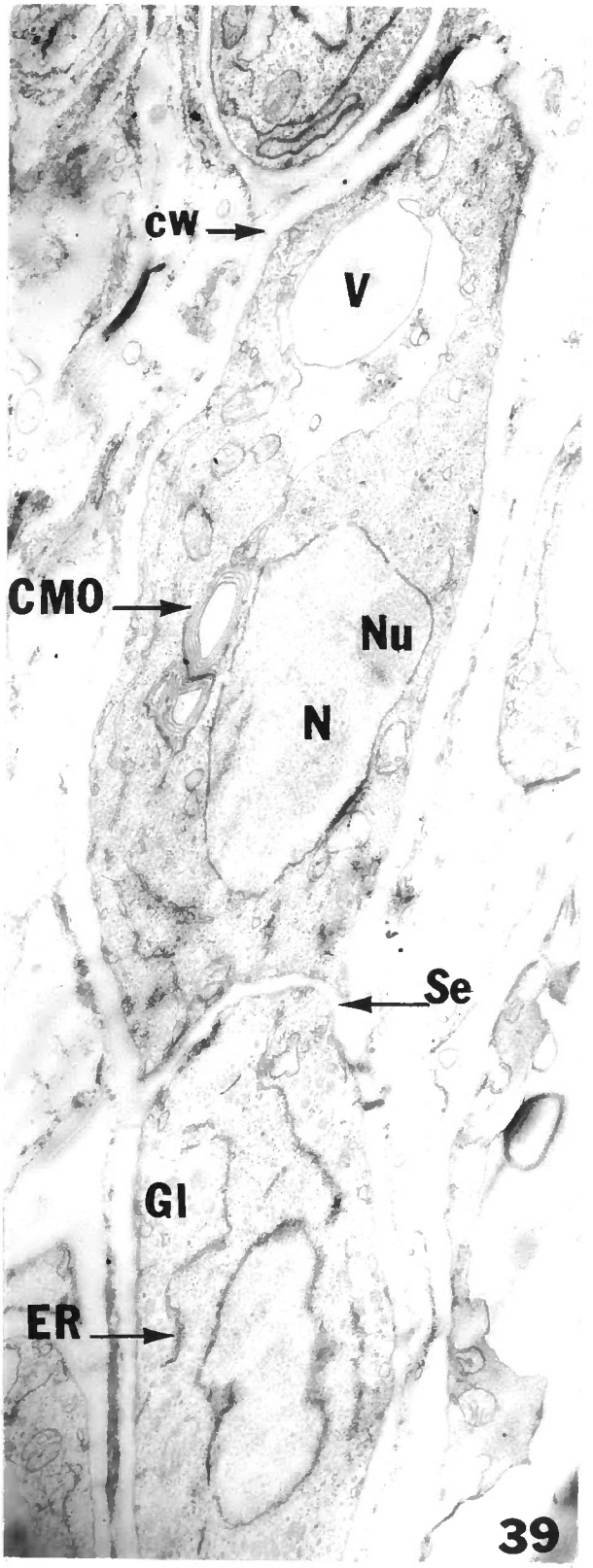
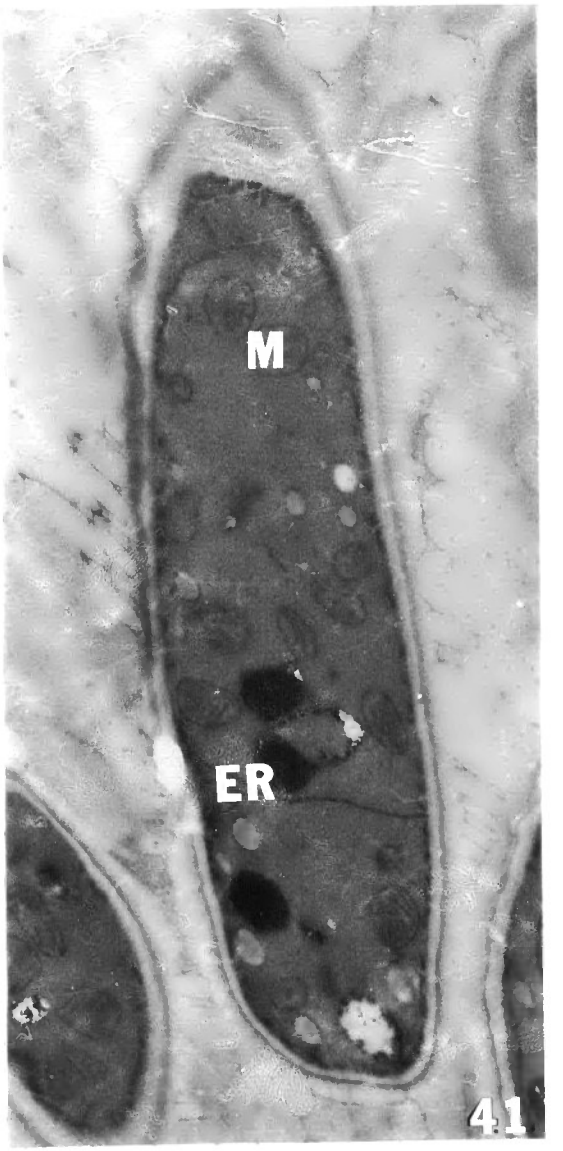

N
M
Se
V
38

Fig. 39. Electron micrograph showing later stage in septum formation. Two-celled conidium shows connection at apical end of conidium to that of conidiophore wall. Note concentric membrane organelles (CMO), nucleus (N), nucleolus (Nu), vacuole (V), glycogen (Gl), common wall (cw), endoplasmic reticulum (ER), and septum (Se). X36,000


cw
V
CMO
Nu
N
Se
Gl
ER
39

Figs. 40-41. TEM views of delimited conidium ER profile at position in cell where septum forms. Note mitochondria (M). X14,560


M
ER
40


M
ER
41

that as the conidium matures it remains attached at its apical end to its original wall. At this stage this wall is regarded as the wall of the conidiophore. In this area the conidiophore wall becomes thickened and has the appearance of a cap-like process over the top of the spore (Fig. 42).

As noted under the light microscope, the protoplast of the developing conidium becomes extended at its apex and the extension becomes the stalk or knob-like process that has been referred to previously as the pedicel. A longitudinal section through the pedicel region is shown in Fig. 43. A thin strand of cytoplasm is present in this region during the early stages of its development (Fig. 39). As the wall thickens at the conidiophore apex the cytoplasmic strands disappear. The dark zone shown in the pedicel region in Fig. 43 provides an indication of initial continuity of the cytoplasm between the conidium and the pedicel.

A fully developed conidium with its pedicel and portions of two of its setulae is shown in Fig. 44. The conidium wall consists of two layers, viz., a thin, electron-dense outer layer, and an electron-transparent inner portion. The original wall that encloses the conidium initial during its early stages of development is fully separate from the wall of the conidium. Careful study of the pedicel region of the spore shown in Fig. 44 reveals that the setulae are derived from the conidiophore wall. As previously demonstrated (Fig. 18) the basal portion of each setula is broad. This part of the setula apparently comes from the thickened inner portion of the conidiophore wall at its apical end and remains attached to the

Fig. 42. Electron micrograph showing thickening of conidiophore wall (CW) at apical end. Note mitochondria (M), nucleus (N), septum (Se), vacuole (V), and membranous material (CMO). X51,000

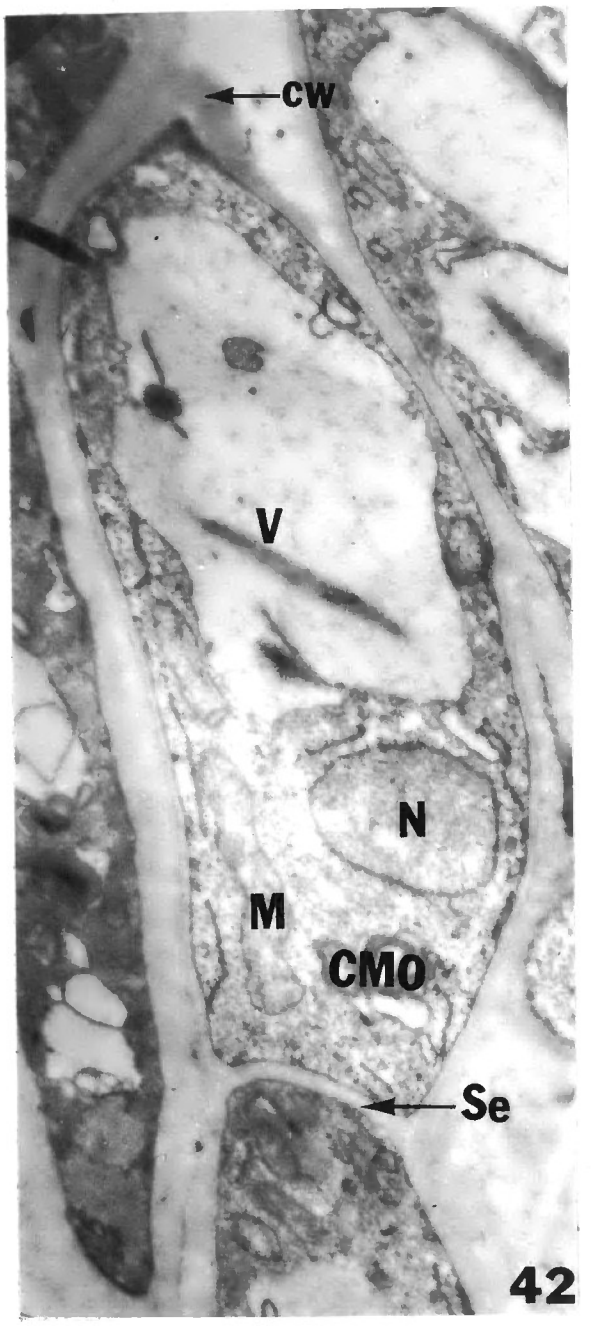

CW
V
N
M
CMO
Se
42

Fig. 43. Electron micrograph showing pedicel (Pe) of spore (SW). Note the relationship of the conidiophore wall (CW) and spore wall to the pedicel. Note basal portion of the setulae (St), and mitochondria (M). X35,000

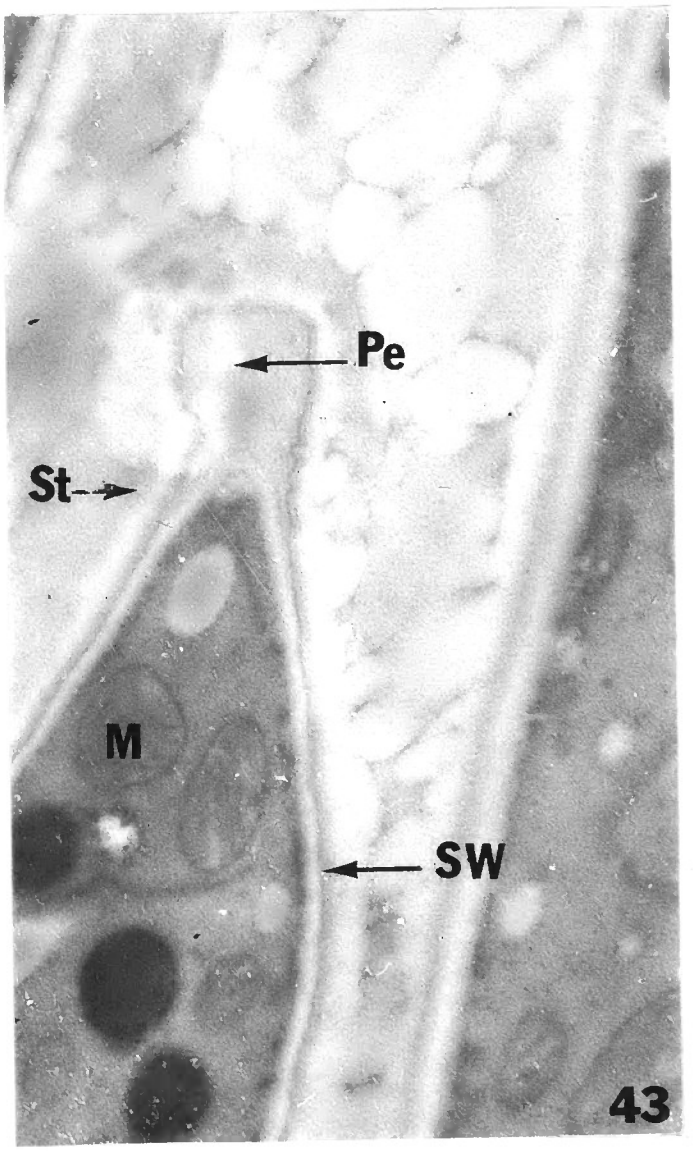
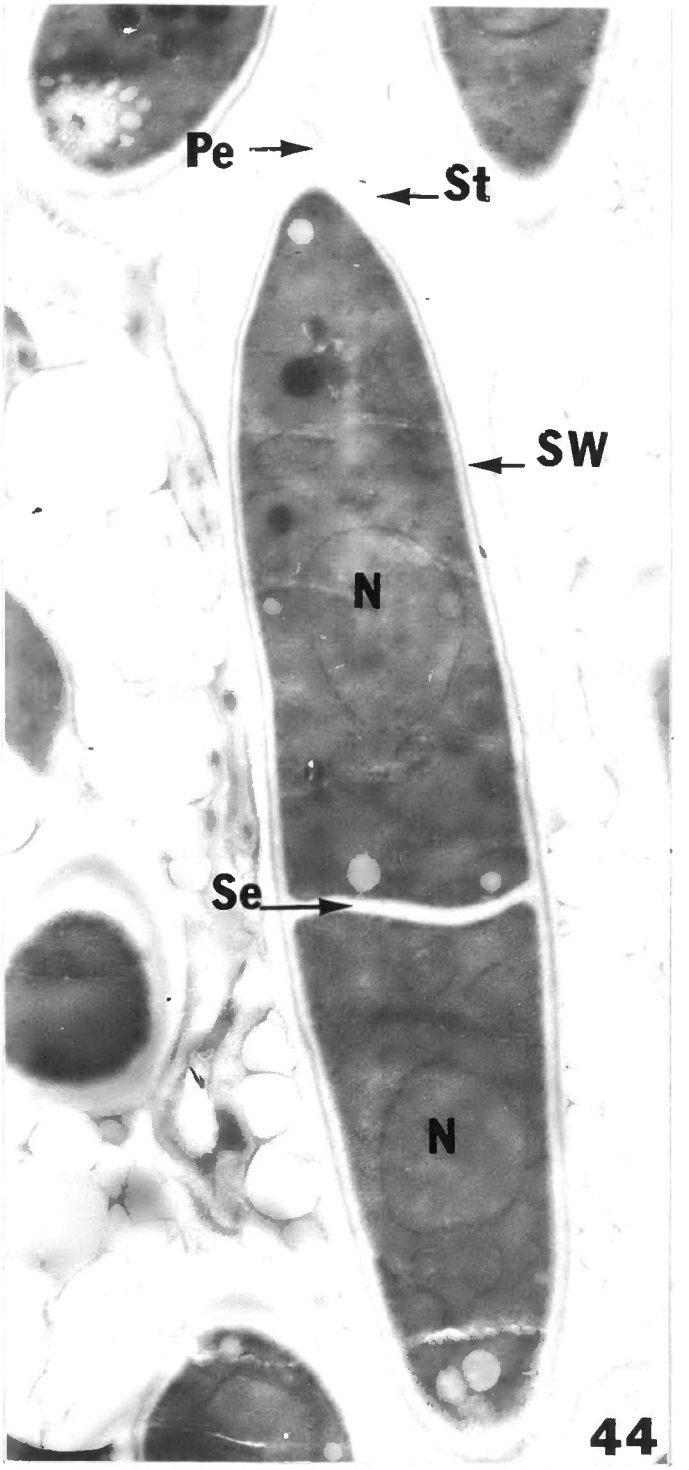

Pe
St
M
SW
43

Fig. 44. Electron micrograph showing mature conidium with septum (Se), nuclei (N), pedicel (Pe), spore wall (SW), and setulae (St). X14,000


Pe
St
SW
N
Se
N
44

conidium by the pedicel.

Cross sections of the pedicel and its relationship to the spore and setulae are shown in Figs. 45-46. Longitudinal sections through setulae are also shown.

Cytoplasmic Features of Conidium Formation

Young conidiogenous cells revealed many irregularly shaped mitochondria, abundant ER, several vacuoles, concentric membrane organelles, vesicles and glycogen masses.

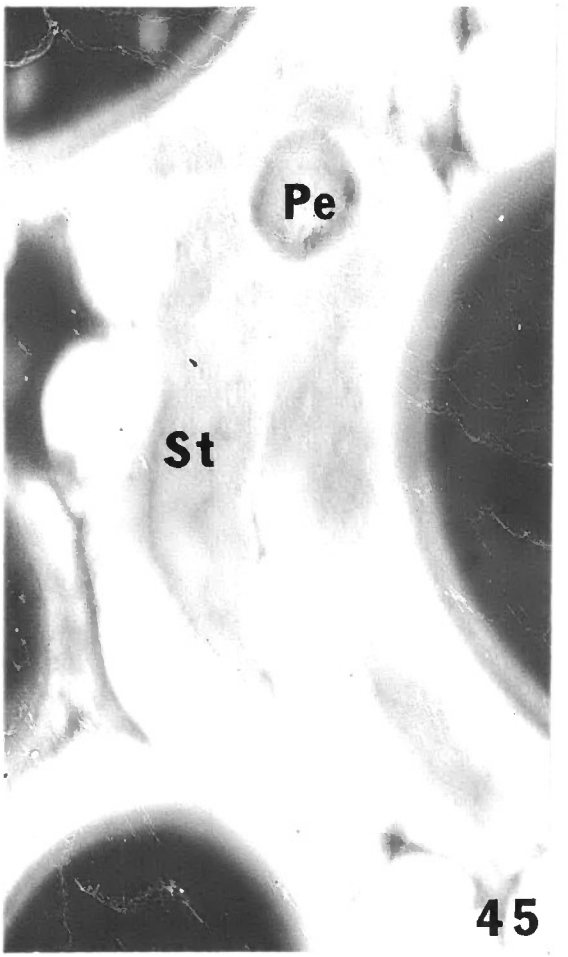
Mitochondria in these cells are round to elongate and contain conspicuous plate-like cristae (Fig. 29). ER profiles varied from short to long and were common (Fig. 34). Vacuoles were small and generally free of contents (Figs. 28-29). Some vacuoles, however, were surrounded by membrane profiles or contained membrane complexes (Figs. 32, 34-35).

Whorls of membranes, as shown in Figs. 28, 30, 34, 36-37, and 39, are clearly evident in several conidiogenous cells. These membranous structures are similar to organelles termed "myelin figures" by several other workers (Carbonell and Pollak, 1962; Moore-Landecker, 1970; Cole and Aldrich, 1971b). Although some investigators have considered myelin figures to be artifacts, Cole and Aldrich (1971b) suggested that myelin-like figures are not artifactual and probably reflect the true cellular condition of those kinds of cells where they have been observed. In the literature differences have prevailed relative to the appropriate name for these membranous organelles. In this study these whorls of membranes will be referred to as "concentric membrane organelles".

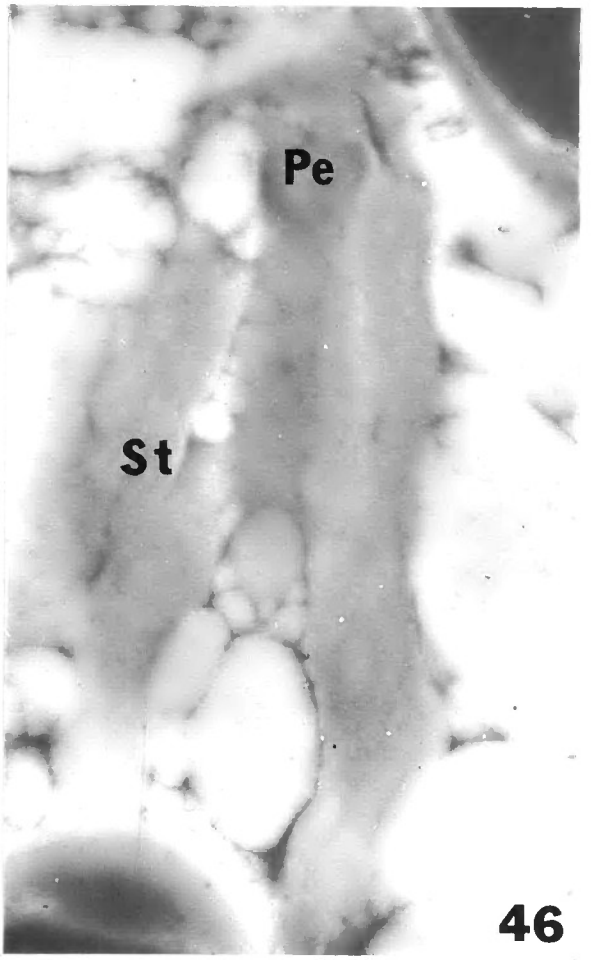
Figs. 45-46. TEM micrographs showing cross section view of pedicel and longitudinal view of setulae.

45. Cross section of pedicel (Pe) and a longitudinal section of setulae (St). X32,000

46. Pedicel (Pe) and setulae (St) at higher magnification. X33,500



45



46

During the early stages of conidiogenesis concentric membrane organelles appear in the basal cell, as shown in Fig. 28. As elongation of this cell progresses, fragmentation of this membrane complex occurs and vesicular elements of various sizes appear (Fig. 30). Further fragmentation of concentric membrane organelles is shown in Fig. 32. An off-center, electron-transparent zone, containing an occlusion, is shown in these organelles in Figs. 28 and 37.

Cantino and Truesdell (1972) stated that technically myelin figures (concentric membrane organelles) should be viewed as "artifacts". Even though they considered them to be artifactual they concluded that they may be significant because they probably represent visible manifestations of intrinsic in vivo alterations in membranes. During developmental stages of Pseudorobillarda conidia, it has been noted that these concentric membrane organelles are commonly present at times when increases in surface area of the plasmalemma may be occurring and during times when additional wall material is being deposited.

Vesicles of various sizes, as shown in Figs. 28, 30, 36, and 39 were observed at most stages of conidiogenesis. Often they appear in positions suggestive of fusion with the plasmalemma (Figs. 28-29, 36, 37). The matrices of vesicles often differed in the spores. Once fusion with the plasmalemma occurred, vesicles appeared to release their contents into the matrix of the developing wall. Vesicles were not observed in mature conidia.

The plasmalemma at some stages appeared to be convoluted (Figs. 34, 36) and resembled the apex of growing hyphal tips. Also associated

with the plasmalemma, at various sites, were parallel segments of ER (Figs. 34-37).

Masses of electron-dense granules, as shown in Figs. 28, 34-36, and 38-39, were observed during all developmental stages. The chemical nature of these granules is unknown since no histochemical studies were conducted. However, similarly appearing electron-dense granules were identified histochemically in a subhymenial cell of Boletus rubinellus as glycogen (McLaughlin, 1971). McLaughlin (1971) stated that glycogen, appearing as aggregates of particles 15 to 30 nm in diameter, were common in fungi as a common storage product.

One or more nuclei (Figs. 34, 39) were commonly noted in conidiogenous cells. A double membrane with well-defined nuclear pores (Figs. 30, 39) was conspicuous around nuclei in these cells.

Fig. 44 shows a mature conidium. The plasmalemma is relatively smooth and vesicles appear to be absent. This absence of a vesicular system suggests that the metabolic rate at this stage is lower and wall synthesis has ceased. Fewer mitochondria, little endoplasmic reticulum, and fewer and smaller vacuoles were noted in mature conidia. A single nucleus is located in each of the cells of the condium and occupies a position in the upper part of each cell. The nucleus is typically spherical.

CHAPTER V

DISCUSSION

Light, scanning, and electron microscopic observations have revealed that the conidia of Pseudorobillarda are phialoconidia that are formed endogenously. Each conidium initial is first enclosed by a cell wall that is common with that of the protoplast of the basal cell, the source of the conidium initial. This original wall becomes the wall of the conidiophore, after spore delimitation, and persists throughout spore maturation.

Associated with spore and paraphysis development with pycnidia of Pseudorobillarda is the elaboration of abundant mucilaginous material. This matrix fills the pycnidium, covers the conidia, and subsequently makes it difficult to observe topographical surfaces when viewed under SEM. SEM micrographs show that this matrix results in spores adhering to each other in clumps and causes, in part, the surface of spores to appear wrinkled. TEM micrographs reveal the mucilaginous matrix as an amorphous, structureless layer that fills in the spaces between conidiogenous cells and paraphyses.

Light and EM observations reveal that the conidium arises from the tips of basal cells. The conidium initial appears as a rounded outgrowth from the top of the basal cell. A continuity of protoplasm and plasmalemma remains between the conidium initial and basal cell until late into conidiogenesis.

It has been observed through TEM that the wall common to the basal cell and the conidium initial has the structure typical of hyphal cells.

It consists of a single layer with an electron-transparent inner portion and a thin, electron-dense outer portion. TEM micrographs reveal that this wall grows at the apical end of the cell until the developing conidium has extended to its full length. During this period of development there is no cell wall deposition around protoplast that represents the initial.

Light microscopy observations have shown that when the conidium initial separates from the protoplast in the basal cell that a thin line is often seen extending upwards to a refractive spot at the tip of the conidiophore. This region is in the part of the developing spore that becomes the pedicel noted in SEM and TEM micrographs. The nature of the small refractive spot is not known. True median sections through this spot were not observed. It bears a slight resemblance to the refractive spot noted by Grove and Bracker (1970) in growing hyphal tips of Gilbertella persicaria. The refractive spot in developing Pseudorobillarda conidia is of a small dimension, however. It probably represents cytoplasmic remnants at the point where the original conidiogenous cell wall, now the conidiophore wall, and the extented cytoplasmic strand remain in contact.

The nature of the pedicel has not been fully resolved. SEM and TEM observations suggest that the pedicel represents an attentuation of the wall at its apical end. Presumably when full conidium delimitation occurs the narrow cytoplasmic strand that remains attached to the conidiophore wall becomes covered by a new wall that forms in the spore between the wall of the conidiophore apex and the cytoplasmic strand. This wall apparently thickens somewhat, the cytoplasm disappears,

and the region of the conidiophore persists as a pointed, knob-like projection to which the setulae adhere. The conidiophore wall breaks towards its basal end when strips of it apparently persist and remain attached to the spore apex. The basal portion of each setula is broad and it tapers rather strongly to a fine and delicate point. The basal part of the setula is apparently derived from the thickened portion of the conidiophore wall at its apical end. TEM and light micrographs show that this region of the conidiophore wall is greatly thickened. Distal from the apex, the conidiophore wall is uniformly thin. The base of the setulae are clearly separate from each other in the mature released conidium. In TEM micrographs the setula is revealed as homogeneous in nature without fibrillar or granular structure. The gross chemical nature of the setulae has not been determined. They are presumed to be mucoid in nature.

Bright-field phase contrast observations have shown that once a conidium has been released, the lower portion of the conidiophore wall persists in association with the basal cell. Subsequent proliferating conidiogenous cells develop within this remnant but within a new common wall. The remnant of the previous conidiophore wall plays no role in the formation of the new conidium.

The time of septum formation during spore ontogeny is apparently not precise. Septum formation may take place at some time during the later stages of spore maturation or it may occur after spore release. The former pattern is probably the more common one. There is some evidence that the portion of the septum is marked initially by an ER segment. In some developing conidium it has been noted that a long ER

profile becomes oriented transversely in the young conidium in the position where septa eventually occur, prior to the actual formation of septa. In fully developed septa cytoplasmic continuity between cells is maintained through a septal pore. Electron-dense inclusions, resembling Woronin bodies, have been noted at these pores. The presence of the Woronin bodies indicates that the septal pore apparatus in Pseudorobillarda may be similar to that typical in other Deuteromycetes known to have ascomycetous affinities (Bracker, 1967). He further states that the presence and morphology of septa and septal pores are useful in establishing phylogenetic relationships among fungi.

The development of various organelles during conidiogenesis has been observed to follow a distinct pattern. Mitochondria are small, irregularly shaped, and abundant during early development of the conidiogenous cell. The mitochondria, in some instances, are more abundant at the conidium apex during elongation and could possibly indicate high metabolic activity. As conidiogenous maturation progresses prominent cristae become evident in the mitochondria. The mature conidia have fewer and smaller mitochondria.

Endoplasmic reticulum is abundant during early conidiogenesis and tubular cisternae are noted at the apex of the early conidiogenous cell. These tubular cisternae are often seen distributed at random along the inner surface of the plasmalemma.

Vesicles have been observed in all stages of conidiogenesis. These structures appear to function in wall formation and plasmalemma surface area increase since they are generally observed during stages when wall material is being deposited and occur in positions which suggest that

they may play a role in deposition. Grove and Bracker (1970) reported that enzymes and/or wall precursor substances may be located within these vesicles.

Configurations with concentric whorls of lamellar membranes were noted in the cytoplasm and in or near the nucleus and vacuoles. These configurations have been termed myelin figures by other workers, but in this study, as indicated previously, they are referred to as concentric membrane organelles. Having observed these structures at different stages of spore development, it appears that they may fragment into vesicular elements. If this is so, then concentric membrane organelles may be involved in the formation of the spore wall and the setulae. As vesicles they may fuse with the plasmalemma and deposit wall material.

Since these structues are widely distributed in the cells, vary in form and size, and occur near the nucleus or in vacuoles, they may play different physiological roles according to their developmental stages and their location. They are not viewed as artifacts, in this study, as they are considered to be by Oláh and Kõhlich (1966) and Cantino and Truesdell (1972). These workers have suggested that these structures are artifacts of fixation.

Some other investigators have maintained that these membrane configurations are regular cellular organelles and have a functional role in the cell (Carbonell and Pollak, 1962; Hashimoto and Yoshida, 1966; Hyde and Walkinshaw, 1966; Pontefract et al., 1969; Smith and Marchant, 1968; Thomas and Isaac, 1967; Moore-Landecker, 1970; Cole and Aldrich, 1971b). These investigators and others have noted that configurations

with concentric whorls of lamellar membranes apparently are of widespread occurrence in fungi.

Mature conidia do not contain the previously mentioned vesicles or concentric membrane organelles. Also the plasmalemma of mature spores is smooth and not convoluted as in the early conidiogenous cells.

The ground cytoplasm of the mature conidium does not appear as electron-dense as that of the conidiogenous cells. This may be due to the massive clusters of glycogen observed in the early developing cells.

Observations made in this study clearly show that findings from the fine structure study of conidiogenesis in Pseudorobillarda differ from light microscopy observations by Cunnell (1958) in regards to setulae formation. Cunnell (1958) indicated that the proximal parts of the setulae are organized within the basal transparent region where they are bent back to join each other and the end of the spore. From this study it is clear that setulae do not originate in this manner.

It can now be stated that Pseudorobillarda, a segregate of Robillarda, differs in three ways: (1) Pseudorobillarda conidia develop endogenously according to Nicot and Rouch (1965), and those of Robillarda develop exogenously. (2) The setulae in Pseudorobillarda originate at the apical end from strips of the conidiophore wall, and the setulae of Robillarda are reported to form as cytoplasmic extensions from the spore proper at its apical end. They are then delimited by a septum. (3) Spores of Pseudorobillarda are enteroblastic phialospores and those of Robillarda are apparently porospores.

CHAPTER VI

SUMMARY

The sequence of events in conidium ontogeny in Pseudorobillarda phragmitis has been studied by light, scanning, and electron microscopy. Conidium development has been traced in order to correlate details on conidium ontogeny in this genus and setulae origin and position on conidia.

1. Light and EM observations reveal that the conidia are enteroblastic and arise apically as outgrowths from basal cells. The protoplasm and plasmalemma of basal cells and conidium initial are in continuity until separated by a hyphal constriction later in conidiogenesis. The spore remains firmly attached, however, to the original wall of the conidiogenous cell at its distal end.

2. Transmission electron micrographs reveal that the conidiophore wall consists of a single-layer which consists of an electron-transparent inner portion and a thin, electron-dense outer portion.

3. In all developmental stages there is an abundant membrane system. As conidial ontogeny progresses, this membrane system decreases. ER material is sparse in mature conidia.

4. Mitochondria are abundant and irregularly shaped. As conidiogenesis progresses they become small and rounded with prominent cristae.

5. Vesicles are present in all stages of conidiogenesis. The mature conidium is void of vesicles. Therefore, they are thought to function in wall formation and cell surface elongation.

6. Concentric membrane organelles are observed after paraformaldehyde fixation and potassium permanganate post-fixation. No concentric membrane organelles were noted in mature conidia. These structures are thought to fragment into vesicular elements that function in spore wall and setula formation.

7. The conidium cell wall has a structure similar to that of the conidiophore cell wall.

8. The pedicel is formed from the spore wall as an apical extension. It is the point of attachment for the setulae.

9. Setulae are attached at the apical end of the spore and represent strips of the conidiophore wall.

10. Bright-field phase contrast studies confirmed that the old conidiophore wall persists as a remnant around the newly proliferating conidiogenous cell. This remnant does not contribute to the development of the new conidiogenous cell.

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